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(54) Title: REGULATED EXPRESSION OF GHRH

(57) Abstract: The present invention provides for a regulated gene expression system for growth hormone releasing hormone ("GHRH") characterized by low basal expression and high specific inducibility. The inducible-expression system includes two expression cassettes. The first expression cassette includes a promoter driving the expression of a molecular switch fusion protein which comprises a DNA binding domain, a transactivation domain and a ligand-binding domain. The fusion protein is characterized by an inability to autodimerize in the absence of an inducer. The second expression cassette includes the gene encoding GHRH controlled by an inducible promoter which is activated by the fusion protein dimerizing in the presence of the inducer and binding to the promoter. The present invention includes therapeutic methods for treating growth hormone-related deficiencies associated with the growth hormone pathway; growth hormone-related deficiencies associated with genetic disease; wasting symptoms associated with burn, trauma, cancer, AIDS, and bone loss, as in elderly, or post-fracture. By administering an exogenously supplied inducer the expression system can be activated and controlled.

REGULATED EXPRESSION OF GHRH

TECHNICAL FIELD

The present invention relates to a regulated gene expression system for growth hormone releasing hormone ("GHRH") characterized by low basal expression and high specific inducibility, and to the use of regulated gene expression for control of GHRH expression in gene therapy.

BACKGROUND OF THE INVENTION

One goal of gene therapy is to deliver genes to somatic tissue in a manner that provides correction of inborn or acquired deficiencies and imbalances. Gene-based drug delivery offers a number of advantages over administration of recombinant proteins. These advantages include: conservation of native protein structure; improved biological activity; prolonged exposure to protein in the therapeutic range; prolonged availability of protein from each administration; avoidance of systemic toxicities; and avoidance of infectious and toxic impurities. Gene therapy is particularly relevant to the provision of hormones such as Growth Hormone Releasing Hormone ("GHRH") in which an extremely short in vivo half-life renders administration of recombinant proteins impracticable.

Growth Hormone ("GH") enhances protein synthesis, lypolysis, and epiphyseal growth. GH increases production of Insulin-like Growth Factor-I (IGF-I) in the liver and peripheral tissues, which further mediates growth. Effective and regulated expression of the GH and IGF-I pathway is essential for optimal linear growth, homeostasis of carbohydrate, protein, and fat metabolism, provides a positive nitrogen balance. GH and IGF-I also have beneficial effects on immune function (LeRoith, D. et al., *Endocrinology* 137:10711079 (1996); Kotzmann, H. et al., *Neuroendocrinology* 60:618-625 (1994)).

GH synthesis and secretion from the anterior pituitary is stimulated by growth hormone releasing hormone (GHRH), a hypothalamic hormone (Muller, E. E., et al. (1999) *Physiol Rev.* 79, 511-607). The GHRH-GH-IGF-I axis undergoes dramatic changes during the aging process and in the elderly (Iranmanesh et al., (1991), *J. Clin. Endocrin. & Metab.* 73:1081-1088; D'Costa A.P. et al., (1993), *J. Reproduction & Fertility Suppl.* 46:87-98,) with decreased GH production rate and GH half-life and a decreased IGF-I response to GH and GHRH stimuli resulting in osteoporosis, increased fat

and decreased lean body mass and tissue function (Corpas et al., (1993), *Endocrine Rev.* 14:20-39).

In addition, genetic disorders of growth have also been ascribed to defects in the GHRH-GH-IGF-I axis, including those of GHRH receptor (Cao et al. (1995) *Pediatr. Res.* 38:962-966), GH gene (Cogan et al. (1993) *J. Clin. Endocrin. & Metab.* 76:1224-1228; Vnencak-Jones et al. (1988) *Proc. Natl. Acad. Sci.* 85:5615-5619), GH receptor (Amselem et al. (1993) *Human Molec. Gen.* 2:355359; Amselem et al. (1991) *Paediatrica Scandinavica Supplement* 377:81-86; Meacham et al. (1993) *J. Clin. Endocrin. & Metab.* 77:1379-1383) and pit-1 (Parks et al. (1993) *Hormone Research* 40:54-61), a pituitary specific transcription factor. In many cases growth retardation is a secondary manifestation of an unrelated primary affection (Turner syndrome, chronic renal failure, ovary resistant syndrome) or the exact cause of growth retardation cannot be established (Parks et al., in Molecular Endocrinology: Basic Concepts and Clinical Correlations (ed. Weintraub, B.D., Raven Press Ltd., New York, (1995) p.473-490). In these cases of growth retardation where the GHRH-GH-IGF-I axis is unaffected, in the elderly, as well as in nonstatural related catabolic conditions (burn, sepsis, trauma associated pathology, chronic obstructive pulmonary disease), GH or GHRH replacement therapy can be effective.

There are a wide-spectrum of clinical conditions, both in children and adults, in which body composition or linear growth (prepubertal patients) is compromised and that respond to GH or GHRH therapy (Ogilvystuart, A. L., et al. (1997) *Clinical Endocrinology* 46, 571-578; Scacchi, M., et al. (1997) *J.Endocrinol.Invest.* 20, 72-76; Thorner, M., et al. (1996) *J Clin.Endocrinol.Metab* 81, 1189-1196). Cachexia, with anorexia, weight loss, muscle wasting, and fatigue, resulting in poor performance status is a common, persistent condition in patients with cancer, AIDS or that have undergone extensive surgery. Barber, M. D., et al. (1999) *Surg.Oncol.* 8, 133-141; Yeh, S. S. and Schuster, M. W. (1999) *Am.J Clin.Nutr.* 70, 183-197. It has been shown that GH and/ or insulin-like growth factor I (IGF-I) therapies are effective in producing a short-term benefit with an anabolic response (Nelson, K. A. (2000) *Semin.Oncol.* 27, 64-68.)

The production of recombinant proteins has provided a useful tool for the treatment of GH-deficiencies in children, the aging population, or as an anabolic drug in burn, sepsis, and AIDS patients. Although GH replacement therapy is used clinically with beneficial effects, this therapy is associated with important disadvantages: side effects

occur frequently, including edema, hypertension, carpal tunnel syndrome, hyperinsulinemia and impaired glucose tolerance (Marcus et al. (1990) *J. Clin. Endocrin. & Metab.* 70:519-527; Salomon et al. (1989) *New Engl. J. Med.* 321:1797-1803); GH must be administered subcutaneously or intramuscularly once a day to three times a week for month, or usually years; insulin resistance and impaired glucose tolerance often result (Angelopoulos, T. J., et al. (1998) *Gerontology* 44, 228-231); in pediatric patients, there is accelerated bone epiphysis growth and closure or slipping of the capital femoral epiphysis (Blethen, S. L. and Rundle, A. C. (1996) *Horm.Res.* 46, 113-116; Blethen, S. L. and MacGillivray, M. H. (1997) *Drug Saf* 17, 303-316); the molecular heterogeneity of circulating GH may have important implications in growth and homeostasis (Satozawa, N., et al. (2000) *Growth Horm.IGF.Res.* 10, 187-192.) It has been suggested that these unwanted pathologies, including insulin resistance, result from the fact that with exogenous GH, the basal levels are raised and the natural GH episodic pulses are abolished.

In lieu of using GH or IGF-I directly, GH secretagogues such as GHRH allow for normal homeostasis of the GH-IGF-I axis by stimulating the pulsatile release of endogenous GH and retaining feedback control of endogenous GH and IGF-I thus avoiding imbalances of and between GH and IGF-I levels. GHRH therapy is expected to be more physiological than GH therapy. GHRH administration permits a degree of feedback, which is totally abolished in the GH therapies. GHRH would be expected to have therapeutic utility in the treatment of cachexia in chronic diseases such as cancer, diabetes, due to growth hormone production abnormalities, enhancement of burn and wound healing, bone healing, retardation of the aging process and osteoporosis. In contrast to recombinant GH, practically no side effects have been reported for GHRH therapies, even at similar dosage (Thorner, M. O., et al. (1986) *Recent Progress in Hormone Research* 42, 589-640). Studies have shown that continuous infusion with GHRH restores a normal GH pattern, with no desensitization of GHRH receptors or depletion of GH supplies in humans, sheep or pigs (Dubreuil, P., et al. (1990) *Journal of Animal Science* 68, 1254-1268; Vance, M. L., et al. (1989) *Journal of Clinical Endocrinology & Metabolism* 68, 22-28; Vance, M. L., et al. (1985) *J.Clin.Invest.* 75, 1584-1590).

Recombinant GHRH is being tested in human clinical trials in the elderly for indications including congestive heart failure, osteoporosis, and improvements in body composition and function in the frail elderly. Recombinant GHRH has been found to

boost the nighttime secretion of growth hormone with concomitant increase in blood IGF-I levels and reduction in body fat. However, current limitations of recombinant GHRH therapy are the high cost of recombinant proteins and the short half-life of the peptides *in vivo* resulting in a requirement for frequent (one to three times a day) intravenous, subcutaneous or intranasal (requiring 300-fold higher dose) administrations (Evans, W. S., et al. (1985) *Journal of Clinical Endocrinology & Metabolism* 61, 846-850; Thorner, M. O., et al. (1986) *Hormone Research* 24, 91-98). Thus, as a chronic therapy, recombinant GHRH administration is not practical.

As an alternative to the use of recombinant proteins, gene therapy provides delivery of genes to somatic tissue in a manner that can provide correction of inborn or acquired deficiencies and imbalances. Gene-based drug delivery offers a number of advantages over administration of recombinant proteins. These advantages include: conservation of native protein structure; improved biological activity; prolonged exposure to protein in the therapeutic range; prolonged availability of protein from each administration; avoidance of systemic toxicities; and avoidance of infectious and toxic impurities.

Using a GHRH injectable DNA plasmid based vector can enhance endogenous GH secretion in vertebrate animals with GH deficiencies in a manner more closely mimicking the natural process and in a less expensive manner than classical therapies. A gene therapy approach will overcome this primary limitation to GHRH use and is preferable, as a single injection into the patient's skeletal muscle may permit physiologic GHRH expression for more then 1 year. Intramuscular injection of DNA vector encoding GHRH can persist for several months to produce sustained levels of GHRH and has been shown by the present inventors to result in enhanced growth. (Draghia-Akli R, et al. (1999) *Nat Biotechnol* Dec;17(12):1179-83; Draghia-Akli R, et al. (1997) *Nat Biotechnol* Nov;15(12):1285-9; WO 99/05300; and WO01/06988, incorporated herein by reference in their entirety.)

However, an overabundance of GHRH can result in acromegaly. Furthermore, it has been suggested that GHRH functions as an autocrine growth factor in some tumors. (Kiaris H., et al. (1999) *Proc Natl Acad Sci U S A* Dec 21;96(26):14894-8). Hence, regulated expression of GHRH is desirable for use in animals under some circumstances and may be required to provide an acceptable safety margin for use in humans.

What is needed for expression of proteins such as GHRH is the ability to closely regulate expression of the introduced gene across a range of administration dosages. In particular, what is needed is a regulated gene expression system having extremely low levels of basal expression of GHRH while retaining high inducibility.

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SUMMARY OF THE INVENTION

The present invention provides an improved molecular-switch, inducible-expression system for regulating the expression of a nucleic acid sequence in gene therapy under conditions in which tight control of expression is of particular importance. In one aspect of the invention, a system is provided wherein expression of the gene to be induced is characterized by low or undetectable expression or biological effect in the absence of the inducer, but in the presence of the inducer, is characterized by efficient induction of expression or biological effect. In another aspect of the present invention, a method is provided that induces a measure of tolerance to transgenic proteins, thus making long-term administration of the protein by gene therapy or recombinant protein possible and effective.

Additional embodiments of the present invention include: (1) a method for treating growth hormone-related deficiencies associated with the growth hormone pathway; (2) a method for treating growth hormone-related deficiencies associated with genetic disease; a (3) a method of treating wasting symptoms associated with burn, trauma, AIDS, or other consumption diseases, including conversion to anabolism from a catabolic state associated with wasting associated with cancer, AIDS, burns, or post-surgery; and (4) a method to prevent or treat bone loss, as in elderly, or post-fracture. All of these methods include the step of introducing a regulated expression vector system into an animal, wherein said expression system can be activated and controlled by administration of an exogenously supplied ligand.

In specific embodiments said vector is selected from the group consisting of a plasmid or a viral vector. The vector may be administered in a simple physiologic solution such as a saline or sugar solution or may be formulated with a liposome, cationic lipid, or a cationic, non-ionic or anionic polymer. In further specific embodiments said vector is introduced into myogenic cells or muscle tissue. In a further specific embodiment said animal is a human, a pet animal, a work animal, or a food animal.

In one embodiment of the invention, the inducible-expression system comprises two nucleic acid or expression cassettes. The first expression cassette includes a promoter driving the expression of a molecular switch protein. In one embodiment the molecular switch is a chimeric or fusion protein. The fusion protein may be comprised of a mutated GAL-4 DNA binding domain characterized by an inability to autodimerize in the absence of an inducer. The fusion protein may further comprise a transactivation domain and a

mutated ligand-binding domain of a steroid-hormone receptor capable of being activated by a non-natural ligand inducer such as mifepristone. In a preferred embodiment, the promoter is a tissue-specific promoter such as α-actin promoter specific for muscle tissues. The first expression cassette may also include 5' untranslated regions, synthetic introns, and poly (A) signals that increase the fidelity of expression of the gene to be induced. The second expression cassette may include a gene controlled by an inducible promoter comprising a GAL-4 DNA-binding site.

In another embodiment of the invention, the inducible expression system is applied *in vivo* to effect expression of a transgene for gene therapy purposes. In an alternate embodiment, the inducible expression system administered to an animal or human in conjunction with electroporation. In an alternate embodiment, the inducible system may be formulated with transfection enhancing or nucleic acid protective compounds and administered with or without electroporation. In addition to using a tightly regulated expression system, a method is provided to minimize potential immune responses of the animal to the transgene or any other introduced nucleic acid and proteins. After administration of the expression system, the induction of the expression system may occur after the animal's initial immune reaction to the injection and electroporation has subsided. For example, a lag time between the administration of the expression system and the inducer may be at least 12 days, more preferably, at least 20 days, or most preferably, greater than 50 days. Furthermore, the method may also include the administration of the inducer using a pulsatile program that further enhances the immunotolerance of the animal.

In another embodiment of the invention, the inducible expression system as introduced in an animal may be characterized by the ability to repetitively effect a biological response using administration of an inducer of the expression system. In a preferred embodiment, the biological response to the inducer is maintained over a period of at least one year using only a single administration of the expression system.

Other and further objects, features and advantages will be apparent from the following description of the presently preferred embodiments of the invention that are given for the purposes of disclosure when taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the nucleic acid structures of an improved expression-regulated system comprising a GENESWITCH® regulator plasmid and an inducible GHRH plasmid according to one embodiment of the present invention. Figure 1 also depicts the interaction between the gene product (i.e. regulator protein) of the GENESWITCH® plasmid and an inducer (e.g. anti-progestin) to induce the expression of GHRH encoded by the inducible GHRH plasmid.

Figure 2 depicts the feedback loops of the GHRH-GH-IGF-I Axis.

Figure 3 depicts the amino acid sequence and structure of the GAL-4 DNA binding domain, amino acid residues 1-93. SEQ. ID. NO. 10.

Figure 4 depicts the consensus intron structure SEQ. ID. NO. 6 and the sequence of a synthetic intron SEQ. ID. NO. 5 according to one embodiment of the present invention.

Figure 5 depicts the sequence of a particular synthetic intron, IVS8 SEQ. ID. NO. 7, according to one embodiment of the present invention.

Figure 6A depicts a schematic representation of relevant regions of an inducible promoter with unique restriction sites indicated. Figure 6B depicts the nucleic acid sequence of the 6X GAL-4/TATA promoter region SEQ. ID. NO. 18, and Figure 6C depicts the nucleic acid sequence of the TATA box, initiation ("inr") region and the UT12 transcription factor binding site, SEQ. ID.NO. 19.

Figure 7 depicts the coding sequences of the molecular switch plasmid pGLV65 (SEQ. ID. NO. 12) and pGS1633 v.4.0 (SEQ. ID. NO. 13), wherein SEQ. ID. NO. 13 has a truncated GAL-4 DNA-binding domain.

Figure 8 depicts the amino acid sequences of the GENESWITCH® regulator proteins (SEQ. ID. NOS. 14 & 15) encoded by the coding sequences depicted in Figure 7. SEQ. NOS. 12 & 13 respectively.

Figure 9 depicts the plasmid map of pGS1633, GENESWITCH® Plasmid Version 4.0 having a chicken skeletal α-actin ("SK") promoter according to one embodiment of the present invention.

Figure 10 depicts the plasmid map of phGHRH1674, an inducible human GHRH encoding plasmid according to one embodiment of the present invention.

Figure 11 depicts the complete nucleic acid sequence of pGS1633, SEQ. ID. NO. 28.

Figure 12 depicts the complete nucleic acid sequence of pGHRH1674, SEQ. ID. NO. 29.

Figure 13 depicts the sequence and components of exemplary synthetic muscle specific promoters, SEQ. ID.NO. 21 and 22.

Figure 14 depicts the *in vivo* activity of an early version of a regulated GHRH expression system.

Figure 15 depicts the *in vitro* activity of a tightly regulated GHRH/GeneSwitch system is active according to one embodiment of the present invention. Myoblasts were transfected with a mixture of GHRH/GeneSwitch in the presence (+MFP) or absence (-MFP) of the inducible drug. The construct coding for *E.coli* beta-galactosidase, βgal , is used as a negative control. As a positive control, cells were transfected with a constitutively active pSP-GHRH construct. Ten micrograms of total RNA were separated, transferred onto a nylon membrane and hybridized with a hGHRH cDNA probe.

Figure 16 depicts the *in vivo* activity of a tightly regulated GHRH/GeneSwitch system is active according to one embodiment of the present invention. A single injection of GHRH/GeneSwitch increases IGF-I serum levels upon activation of the system with MFR. Plasma IGF-I level after direct intramuscular injection of pSP-GHRH constructs, ANOVA for the entire + MFP series *$p<0.03$.

Figure 17 depicts weight and pituitary organ change upon chronic MFP induction. Figure 17A depicts average weight increase in injected mice upon chronic activation of the

GHRH/GeneSwitch system, * $p < 0.027$. Figure 17 B depicts pituitary weight / total body weight in +MFP injected animals, * $p < 0.035$.

Figure 18 depicts body composition changes in chronically induced GHRH/GeneSwitch mice. Figure 18A depicts the significant increase in lean non-bone mass, * $p < 0.022$. Figure 18B depicts the significant decrease in fat body mass/ total weight in induced animals, * $p < 0.05$.

Figure 19 depicts body composition changes in chronically induced GHRH/GeneSwitch mice. Figure 19A depicts that bone area is increased by PIXImus, *$p < 0.0006$. Figure 19B depicts that bone mineral content is increased in induced animals, * $p < 0.002$.

Figure 20 depicts GHRH sequences. Figure 20A depict one embodiment of a codon optimized human 1-40 aa GHRH. Figure 20B depicts the amino acid sequences of several alternate GHRH species.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

With long-term expression of gene products, and particularly in circumstances in which physiological effects are unpredictable, or are associated with adverse effects or toxicities, delivery of certain genes or gene products may require the ability to closely control expression of transfected genes from outside the body.

Secretion of Growth Hormone ("GH") is regulated as part of the Growth Hormone Releasing Hormone - Growth Hormone - Insulin Growth Factor I axis ("GHRH-GH-IGF-I axis") as depicted in Figure 2. GH synthesis and secretion from the anterior pituitary is stimulated by GHRH and inhibited by somatostatin, both hypothalamic hormones. GH increases production of IGF-I, primarily in the liver, and possibly other target organs. IGF-I and GH, in turn, feedback on the hypothalamus and pituitary to inhibit GHRH and GH release. Effective and regulated expression of GH and IGF-I is essential for optimal linear growth, homeostasis of carbohydrate, protein, and fat metabolism, provides a positive nitrogen balance, and has beneficial effects on immune function.

By GHRH it is meant a protein having GHRH activity that is able to regulate the GH synthesis and release. There are issued patents that describe GHRH analog proteins (U.S. Pat. Nos. 5,847,066; 5,846,936; 5,792,747; 5,776,901; 5,696,089; 5,486,505; 5,137,872; 5,084,442; 5,036,045; 5,023,322; 4,839,344; 4,410,512; RE33,699) or

synthetic or naturally occurring peptide fragments of GHRH (U.S. Pat. Nos. 4,833,166; 4,228,158; 4,228,156; 4,226,857; 4,224,316; 4,223,021; 4,223,020; 4,223,019) for the purpose of increasing release of growth hormone. A GHRH analog containing the following mutations has been reported (U. S. Patent No. 5,846,936): Tyr at position 1 to His; Ala at position 2 to Val, Leu, or others; Asn at position 8 to Gln, Ser, or Thr; Gly at position 15 to Ala or Leu; Met at position 27 to Ile or Leu; and Ser at position 28 to Asn.

The GHRH of the subject invention can be any animal GHRH or GHRH analoque having GHRH activity such as for example the GHRH species of SEQ.ID. 31 (human 44 aa GHRH), 32 (humans 40 aa GHRH), 33 (porcine 40 aa wild type), and including protease resistant variants such as for example the GHRH species of SEQ.ID. 34 (super-porcine HV as disclosed in WO 01/06988). For gene therapy purposes the sequence encoding GHRH can be a native sequence or can be codon optimized such as for example the human 1-40 aa codon optimized sequence of SEQ.ID.NO. 30.

Ideally, control of GHRH expression via a "molecular switch" should allow specificity, selectivity, precise timing and level of expression, safety, and rapid clearance of the triggering compound. According to one aspect of the present invention, a system for regulating gene expression is generally depicted in Figure 1 using the GHRH gene as an example.

In one embodiment, a non-viral GHRH gene therapy for the regulation of growth and body composition was developed using a mifepristone ("MFP") dependent GeneSwitch™ technology. The system consisted of two plasmids, one encoding for a chimeric GeneSwitch™ transactivator, and the other for an inducible growth hormone releasing hormone (GHRH). In one example demonstrating the effectiveness of the system, adult SCID mice were injected into the tibialis anterior with either both constructs, only one component of the system, a control beta-galactosidase system, or a constitutively active construct (n=20/group, 10 micrograms/animal). The administration of the GHRH gene was in conjunction with electroporation. Upon administration of MFP for 4 consecutive days, significant up-regulation of IGF-I levels was obtained. IGF levels following administration of both GeneSwitch™ and GHRH plasmid followed by MFP were 1797.28 ± 164.96 ng/ml, versus 1100.86 ± 33.67 ng/ml pre MFP levels in the same group of animals, $p < 0.0006$, 1086.78 ± 65.34 ng/ml in control (beta-galactosidase) injected animals, $p < 0.0007$, 1171.79 ± 42 ng/ml in animals injected with the two plasmids, but without MFP, $p < 0.002$, and animals injected with the constitutively active

construct 1374.22 ± 83.8 ng/ml, $p < 0.03$). IGF-I levels returned to baseline 7 days after MFP was withdrawn. Four rounds of induction were performed to 115 days after injection with similar results. Starting with day 125, the animals were induced with MFP for 24 days and then analyzed by DEXA and for analysis of body composition. Total body weight of chronically MFP induced animals was increased ($p < 0.027$). The weight gain was restricted to lean body mass, while fat was significantly decreased ($p < 0.05$). Pituitary weight was significantly increased, with pituitary weight/ total body weight $7.35x10^{-5} \pm 3.1x10^{-6}$ in MFP induced animals, versus $6.2x10^{-5} \pm 4.6x10^{-6}$ in β-gal controls, $p < 0.035$. Bone mineral area, content and density were significantly increased in treated animals compared with controls. This data supports the use of regulated GHRH to efficiently to deliver growth hormone releasing hormone as adjuvant to enhance or support an anabolic state, as in the treatment of burn, sepsis, large surgery or AIDS, or in the elderly.

The "molecular switch" expression system is generally comprised of two nucleic acid or expression cassettes: (1) a molecular switch or otherwise called a GENESWITCH® plasmid, and (2) an inducible gene plasmid (e.g., Inducible GHRH plasmid). Although Figure 2 suggests that the two nucleic acid cassettes are carried on two different plasmid vectors, the two nucleic acid cassettes may also be combined in a single plasmid vector or a single viral vector having both nucleic acid cassettes.

The term "molecular switch plasmids," as used herein, refers to plasmids encoding chimeric transcriptional regulator or "molecular switch" molecules or proteins having, but not limited to: 1) a sequence specific DNA binding domain (DBD) such as for example the GAL-4 DBD; 2) a mutated steroid receptor ligand binding domain such as for example a human progesterone receptor ligand binding domain having a C-terminal deletion of about 19-66 amino acids wherein the mutant may be activated in the presence of an antagonist for the naturally occurring or wild-type progesterone receptor; and 3) a transactivation domain, such as for example the herpes virus VP-16 or NFκB p65 transactivation domain. The transactivation domains may also be selected from a number of other transactivation domains known to those of skill in the art, such as for example, TAF-1, TAF-2, TAU-1, and TAU-2.

The term "GENESWITCH®" is a registered trademark of Valentis, Inc. and is used to identify "molecular switch plasmids," "molecule switch" proteins or molecules,

and expression systems generated by Valentis, Inc. The prefix "pGS" is abbreviation identifying GENESWITCH® plasmids.

Figure 1 generally depicts the interaction of the molecular-switch plasmid and proteins with the inducible gene plasmid. In general, the first expression cassette contained in the GENESWITCH® plasmid may comprise a promoter driving the expression of a fusion or chimeric protein. The promoter may be any promoter such as a CMV promoter or a tissue-specific promoter for expression in an animal cell. A preferred promoter for use with one aspect of the invention is a muscle-specific promoter with advantages as will be discussed below. The fusion or chimeric protein expressed from the fusion or chimeric gene generally comprises three structural domains represented by GAL-4, hPR LBD, and p65 in the GENESWITCH® plasmid in Figure 1. These three domains also correlate to the functional domains of the fusion protein.

For example, GAL-4 represents nucleic acid sequence correlating to the GAL-4 DNA-binding domain responsible for the interaction or binding of the fusion protein to the 6X GAL-4 promoter in a second nucleic acid cassette, depicted as the inducible EPO plasmid. p65 represents nucleic acid sequence correlating to the transcription regulatory domain of the NFκb p65 protein.

The hPR LBD correlates to the ligand-binding domain of the fusion protein, which is responsible for the interaction of the fusion protein with a ligand represented by an oral anti-progestin in Figure 1. In a specific example, the ligand-binding domain (LBD) is derived from the amino acid sequence correlating to the ligand-binding domain of human progesterone receptor (hPR), a receptor in the steroid-receptor family. As will be discussed in greater details below, the amino acid sequence in the LBD of hPR may be mutated to result in a mutated hPR LBD (or, more generally, a mutated steroid-receptor LBD) that selectively binds to the anti-progestin instead of progestin, the natural ligand/agonist of the progesterone receptor. Through a mutated hPR LBD, the fusion protein may, thus, be selectively activated by an anti-progestin, instead of the naturally occurring progestin.

As shown generally in Figure 1, when the anti-progestin binds to the fusion protein expressed from the GENESWITCH® plasmid, the fusion protein is activated and forms a dimer complex. The dimer/anti-progestin complex, in turn, binds to the promoter of the inducible plasmid and transactivates the transcription of the gene. It should be noted that the specific nucleic acid structures depicted in the two nucleic acid cassettes in Figure 1

are provided as examples, and various modifications can be made to achieve a similarly tightly regulated expression system.

For example, the transregulatory domain represented by p65 may be substituted with various other transregulatory domains such as VP-16, TAF-1, TAF-2, TAU-1, TAU-2 and any other nucleic acid/amino acid sequence having a transcription regulatory function. The DNA-binding domain and the corresponding 6x GAL-4 binding site in the inducible gene plasmid should not be seen as being limited to the GAL-4 DNA-binding domain. Other DNA binding domains may also be used such as known DNA binding domains of the steroid-receptor family (e.g., glucocorticoid receptor, progesterone receptor, retinoic acid receptor, thyroid receptor, androgen receptor, ecdysone receptor) or other cellular DNA binding proteins such as the cAMP Response Element Binding protein (CREB). The GAL-4 DNA-binding domain is preferred because it allows for greater control and selectivity of gene activation using this expression system in mammalian cells.

The steroid-receptor family of gene regulatory proteins is also ideal for the construction of molecular switches. Steroid receptors are ligand activated transcription factors whose ligands can range from steroids to retinoids, fatty acids, vitamins, thyroid hormones, and other presently unidentified small molecules. These compounds bind to receptors and either up-regulate or down-regulate the expression of steroid-regulated genes. The compounds are usually cleared from the body by existing mechanisms and are usually non-toxic. The term "ligand," as used herein, refers to any compound or molecule that activates the steroid receptor, usually by interaction with (binding) the ligand-binding domain (LBD) of the steroid receptor.

The term "steroid-hormone receptor" as used herein refers to steroid-hormone receptors in the superfamily of steroid receptors, some of which are known steroid receptors whose primary sequence suggests that they are related to each other. Representative examples of the steroid-hormone receptors include the estrogen, progesterone, glucocorticoid-α, glucocorticoid-β, mineralocorticoid, androgen, retinoic acid, retinoid X, Vitamin D, COUP-TF, ecdysone, Nurr-1 and orphan receptors. The receptors for hormones in the steroid/thyroid/retinoid supergene family, for example, are transcription factors that bind to target sequences in the regulatory regions of hormone-sensitive genes to enhance or suppress their transcription. These receptors have evolutionarily conserved similarities in a series of discrete structural domains, including a

ligand-binding domain (LBD), a DNA binding domain (DBD), a dimerization domain, and one or more trans-activation domain(s).

Various mutations or changes in the amino acid sequences of the different structural domains may be generated to form a mutated steroid receptor or, more specifically, mutated steroid-hormone receptor. The term "mutated steroid receptor," "modified steroid receptor," or a "mutated steroid-hormone receptor" or "modified steroid-hormone receptor," as used herein is a steroid receptor or steroid-hormone receptor that has been mutated in its amino acid sequences such that the mutated form is capable of preferentially binding to a non-natural or non-native ligand rather than binding to the wild type, or naturally occurring, hormone receptor ligand. Usually this mutation is generated in the ligand-binding domain of the steroid receptor and may be denoted as "mutated steroid-receptor LBD." A mutated steroid receptor has the property to activate transcription of a desired gene (such as a gene encoding erythropoeitin) in the presence of an antagonist for a wild type steroid hormone receptor protein.

Normally, a non-natural or non-native ligand may act as an antagonist or may have an antagonist effects to a wild-type steroid receptor or steroid-hormone receptor. "Antagonist" as used herein is a compound that interacts with or binds to a native steroid hormone receptor and blocks the activity of the agonist of the native steroid hormone receptor. "Agonist" as used herein is a compound that interacts with the wild type steroid hormone receptor to promote a transcriptional response.

For example, progesterone or progestin is an agonist for the progesterone receptor because progesterone normally binds to the progesterone receptor to activate the transcription of progesterone-regulated genes. Compounds, which mimic progesterone, would also be defined as progesterone receptor agonists. Mifepristone (MFP) or otherwise known as RU486 is a non-natural ligand that also binds to the progesterone receptor and competes with progesterone for binding. Although under certain special circumstances, MFP may slightly activate certain progesterone-regulated genes through the progesterone receptor, the amount of activation is minimal when compared to the major activity of MFP, which is to block the activation of the progesterone receptor by progesterone. Hence, in the presence of progesterone and the progesterone receptor, MFP exerts an antagonistic effect on the progesterone receptor because it blocks the normal activation of the receptor by progesterone.

The progesterone receptor may be mutated, e.g. in the ligand-binding domain of the progesterone receptor, such that it only binds to MFP and not to progesterone. The mutation of the ligand-binding domain of progesterone receptor may be such that binding of the MFP may actually activate the progesterone receptor under typical cellular conditions. When a mutated PR LBD, or more generally any other mutated steroid-receptor LBD, is combined as a fusion protein with a particular DNA-binding domain such as the GAL-4 DNA binding domain, binding of MFP selectively activates the fusion protein to transactivate gene expression driven by a promoter recognized by the DNA-binding domain. Thus, the mutated steroid receptor of the subject invention is not activated in the presence of agonists for the native receptor, but instead the mutated steroid receptors may be activated in the presence of "non-natural ligands."

The term "non-natural ligands" or "non-native ligands" refers to compounds that are normally not found in animals or humans and that bind to the ligand-binding domain of a receptor. Examples of non-natural ligands and non-native ligands are anti-hormones that may include without limitation the following:

11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-propinyl-4, 9-estradiene-3-one (RU38486 or Mifepristone);

11β-(4-dimethylaminophenyl)-17α-hydroxy-17β-(3-hydroxypropyl)-13 α methyl-4, 9-gonadiene-3-one (ZK98299 or Onapristone);

11β-(4-acetylphenyl)-17β-hydroxy-17α-(1-propinyl)-4,9-estradiene-3-one (ZK112993);

11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(3-hydroxy-1(Z)-propenyl-estra-4,9-diene-3-one (ZK98734);

(7β, 11β, 17β)-11-(4-dimethylaminophenyl)-7-methyl-4',5'-dihydrospiro [ester-4,9-diene-17,2' (3'H)-furan]-3-one (Org31806);

(11β ,14β, 17α)-4', 5'-dihydro-11-(4-dimethylaminophenyl)-[spiroestra-4, 9-diene-17,2'(3'H)-furan]-3-one (Org31376); and

5-α-pregnane-3,2-dione.

"Mutant," "mutation," "mutated," "modified," or "modification" refers to an alteration of the primary sequence of a receptor or any other gene or protein such that it differs from the wild type or naturally occurring sequence. For example, a mutant, mutated or modified steroid-hormone receptor protein as used in this disclosure can be a

mutant of any member of the steroid-hormone receptor superfamily. For example, a steroid receptor can be mutated by addition of amino acid(s), substitution of amino acid(s) or deletion of amino acid(s). Preferably, the deletion of the amino acids occur on the carboxy terminal end of the protein. Generally, a deletion of from about 1 to about 120 amino acids from the carboxy terminal end of the protein provides a mutant useful in the present invention. A person having ordinary skill in this art will recognize, however, that a shorter deletion of carboxy terminal amino acids will be necessary to create useful mutants of certain steroid hormone receptor proteins. For example, a mutant of the progesterone receptor protein will contain a carboxy terminal amino acid deletion of from about 1 to about 60 amino acids. In another embodiment, 19 carboxy terminal amino acids are deleted from the progesterone receptor protein.

Furthermore, a mutated steroid-hormone receptor LBD may be selected based on the ability of an antagonist for the wild-type steroid-hormone receptor to activate the mutant receptor even in the presence of an agonist for the wild-type receptor. Thus, in the case of the progesterone receptor, progesterone is the normal ligand and functions as a strong agonist for the receptor. The anti-progestin, mifepristone (RU486), is a non-natural or non-native ligand for the progesterone receptor. Mifepristone (MFP) is considered an "anti-progestin" because, although it is able to exert a slight agonist effect on the wild-type progesterone receptor, MFP inhibits the strong agonistic effects of progesterone. Thus, MFP may be considered an "antagonist" for the wild-type progesterone receptor when in the presence of the normal agonist, i.e. when both MFP and progesterone are together in the presence of the wild-type progesterone receptor. In contrast, in one example of a mutated steroid-hormone receptor according to the invention, the mutated progesterone receptor is not activated by progesterone (agonist for the wild type receptor) but is activated in the presence of MFP ("antagonist" for the wild type receptor). In addition, progesterone is not able to block the activation of the mutated steroid-hormone receptor by MFP. Thus, the mutated receptor may be characterized as activated when bound to an antagonist (MFP) for the wild-type receptor even in the presence of an agonist (progesterone) for the wild-type progesterone receptor.

Further examples of mutated and modified steroid-hormone receptor for used with the current invention are described in, for example: (1) "Adenoviral Vector-Mediated Delivery of Modified Steroid Hormone Receptors and Related Products and Methods" International Patent Publication No. WO0031286 (PCT/US99/26802); (2) "Modified

Glucocorticoid Receptors, Glucocorticoid Receptor/ Progesterone Receptor Hybrids" International Patent Publication No. WO9818925 (PCT/US97/19607); (3) "Modified Steroid Hormones for Gene Therapy and Methods for Their Use" International Patent Publication No. WO9640911 (PCT/US96/0432); (4) "Mutated Steroid Hormone Receptors, Methods for Their Use and Molecular Switch for Gene Therapy" International Patent Publication No. WO 9323431 (PCT/US93/0439); (5) "Progesterone Receptors Having C-Terminal Hormone Binding Domain Truncations", U.S. Patent No. 5,364,791; (6) "Modified Steroid Hormone Receptors, Methods for Their Use and Molecular Switch for Gene Therapy" U.S. Patent No. 5,874,534; (7) "Modified Steroid Hormone Receptors, Methods for Their Use and Molecular Switch for Gene Therapy" U.S. Patent No. 5,935,934, (8) "Improved System for Regulation of Transgene Expression", U.S. Patent Application No. 60/278,281 all of which are incorporated herein by reference in their entirety, including any drawings.

Although the examples provided in this disclosure use plasmid-based mutated steroid hormone receptor system, viral-based versions of a mutated steroid hormone receptor system according to the present invention may also be used to regulate gene expression *in vitro* or *in vivo*. There are several specific examples of the use of early versions of inducible transcriptional regulator system in viral vectors. For example: (1) the positive and negative regulation of gene expression in eukaryotic cells with an inducible transcriptional regulator is described in Wang, et al., Gene Therapy, 4: 432-441,1997; and (2) drug inducible transgene expression in brain using a herpes simplex virus vector is described in Oligino, et al., Gene Therapy, 5: 491-496,1998. These above examples may be applied to provide for viral-based regulated gene-expression systems according to the present invention.

To improve the tightness of regulated expression, several aspects of the GENESWITCH® expression cassette and the inducible therapeutic molecule expression cassette were modified. First, the transcribed portion of the GENESWITCH® expression cassette was modified to include post-transcriptional elements (5' UTR, synthetic intron and poly(A) signal) that improve the level and fidelity of transgene expression. Second, the structure of the GENESWITCH® regulatory protein was modified. The regulator protein appears to have a propensity, in the absence of ligand, to form dimers that could bind GAL-4 sites in the inducible promoter and thereby partially activate transcription. To minimize this possibility, truncated or mutation on the GAL-4 domain of the regulator

protein may be made such as deleting from the C-terminal portion of the GAL-4 DBD, about 20 residues, thereby reducing the length of a coiled-coil structure that contributes to GAL-4 homodimer formation. GENESWITCH® regulator protein v.4.0 embodies this modification. Third, the promoter of the GENESWITCH® expression cassette may be replaced with a tissue-specific promoter such as avian skeletal α-actin promoter, which is muscle-specific.

The inducible therapeutic molecule expression plasmid may be also that modified in the core region of the inducible promoter. It has been determined that a deletion in the transcription initiation region of the inducible plasmid can reduce the intrinsic activity of the promoter by approximately 10-fold without impairing its ability to be induced. It should be noted that the modifications described above may be employed independently or in combination with each other depending on the desired effect.

In one embodiment of the present invention, a molecular switch protein comprising a chimeric receptor having a mutated progesterone-receptor ligand-binding domain, a truncated GAL-4 DNA binding domain, and a VP16 or p65 transregulatory domain is disclosed. The p65 transregulatory domain is part of the activation domain of the human p65 protein, a component of the NFκB complex. By replacing VP16 with a variety of human-derived activation domains such as, for example, residues 286-550 of the human p65, the potent inducibility of the chimeric receptor can be retained while "humanizing" the protein or reducing the potential for a foreign protein immune response due to the viral VP16 component. In the presence of the anti-progestin MFP (RU486), this chimeric regulator binds to a target nucleic acid sequence containing a 17mer GAL-4 binding site, and results in an efficient ligand-inducible transactivation of the target gene downstream of the GAL-4 binding site. The modified steroid-hormone ligand-binding domain of the receptor protein may also be modified by deletion of carboxy terminal amino acids, preferably, from about one to one hundred-twenty carboxy terminal amino acids. The extent of deletion desired can be modulated according to conventional molecular biological techniques to achieve both selectivity for the desired ligand and high inducibility when the ligand is administered. In one embodiment, the mutated steroid hormone receptor LBD is mutated by deletion of about one to about sixty carboxy terminal amino acids. In another embodiment forty-two carboxy terminal amino acids are deleted. In yet another embodiment, having both high selectively and high inducibility, nineteen carboxy terminal amino acids are deleted.

As used herein, the GAL-4 DNA Binding Domain ("GAL-4 DBD") refers to amino acids 1 – 93 of the N-terminal DNA binding domain of GAL-4 as shown in Fig. 5 SEQ. ID. NO. 10. As used herein, a "modified GAL-4 DBD" or mutated GAL-4 DBD refers to a GAL-4 DBD that has a mutation in the primary amino acid structure, or to a amino acid sequence derived from the GAL-4 DBD, that retains the ability to bind to the canonical 17-mer binding site, CGGAAGACTCTCCTCCG, (SEQ.ID.NO. 9), but no longer has ability to form a helical tertiary structure needed for autodimerization. In one example, a deletion of a region represented by amino acids 75 to 93 of the native GAL-4 DBD as depicted in Figure 5, provides for a modified or mutated GAL-4 DBD that when combined with the GENESWITCH® regulator protein decreases the basal expression of GHRH from an inducible expression plasmid. Other mutations, including substitutions (changes in the amino acid sequence) or deletions may also be made to the region spanning amino acid sequence 54-74 of the GAL-4 DBD as shown in Figure 5. For example, a deletion of amino 54-64, or 65-75 may be made such that autodimerization through the coiled coil region is minimized.

In one example, an optimized transgene regulation system is disclosed below that meets desired criteria for a robust system. In particular, an improved regulated muscle-specific GHRH/GENESWITCH® system disclosed in one embodiment herein provides undetectable biological effect from a pharmacological dose of the introduced GHRH transgene in the absence of inducer. By "biological effect" it is meant that, although it may be possible to detect the production of messenger RNA by ultra sensitive assays such by Polymerase Chain Reaction ("PCR"), no physiologic effect, such as for example in the case of GHRH, no rise in the level of IGF-I, is observed.

On the other hand, the improved system responded to doses of MFP as low as 0.01 mg/Kg. Responsiveness to low doses of MFP is highly favorable; especially since chronically administered 25 mg doses (0.25-0.5 mg/Kg) are well tolerated in humans.

The improved system may also have several optional components that permit advantages over existing systems. First, the present improved GENESWITCH® regulator protein is mostly humanized (86%) (amino acid sequences derived from human proteins except for the GAL-4 DNA-binding domain); thus reducing the potential for long-term responsiveness of the system in immune-competent recipients.

Second, exogenous control of expression in the present system having undetectable baseline expression may also permit a lag time prior to first induction by MFP or other

inducer. The length of the lag period between plasmid delivery and the first induction of transgene expression permits reduction in the potential for developing immune responses to the expressed transgene. Delivery of plasmids with electroporation, for example, may be associated with transient inflammation and cellular infiltration that are able to activate dendritic cell maturation. Foreign transgene expression that is induced after inflammation at the muscle site has subsided avoids transgene expression in a hyperinflammatory environment. Third, use of a muscle-specific promoter may also provide low level GENESWITCH® protein production and may minimize expression in non-muscle cells.

The term "expression cassette" or "nucleic acid cassette," as used herein refers to the combination of nucleic acid sequences involved in expression of a particular functional product. This functional product is typically a protein although it could also be a nucleic acid such as for example, an RNA molecule such as a ribozyme or antisense RNA. The expression cassette may also be comprised of a number of non-coding elements in addition to sequences encoding a product such as a protein. Non-coding elements are nucleic acid sequences bounded or defined by consensus sequences or having a contextual location identifiable or recognized by those of skill in the art. A "5' untranslated region" or "5' UTR" refers to a sequence located 3' to promotor region and 5' of the downstream coding region. For example, the 5' end of the 5' UTR is typically defined as the transcription start site. Although the start of transcription may not be precisely known, it is often estimated to be approximately 30 base pairs 3' of the end of the TATA box. The 3' end of the 5'UTR would be defined as the base immediately 5' to the start codon (ATG). Thus, such a sequence, while transcribed, is upstream of the translation initiation codon and therefore is not translated into a portion of the polypeptide product. Such a 5' UTR may also have an intron within it. In one embodiment of the present invention, the expression cassette includes: promoter sequences, transcription start sequences, 5' untranslated ("5' UTR") sequences, coding sequences from a start codon through a stop codon, and 3' untranslated sequences ("3' UTR") including polyadenylation sequences. As used herein the 5' UTR may include one or more functional non-coding elements able to increase the level and fidelity of expression. As used herein, the 5' UTR may include intron sequences that are transcribed but spliced out of the mature messenger RNA ("mRNA"). The expression cassette may positionally and sequentially oriented in a vector with other necessary elements such that the nucleic acid in the cassette can be transcribed and, when necessary, translated in eukaryotic cells.

The term "intron" as used herein refers to a sequence encoded in a DNA sequence that is transcribed into an RNA molecule by RNA polymerase but is spliced from the mature messenger RNA. A "synthetic intron" refers to a sequence that is not initially replicated from a naturally occurring intron sequence and generally will not have a naturally occurring sequence, but will be removed from an RNA transcript during normal post-transcriptional processing. Such synthetic introns can be designed to have a variety of different characteristics, in particular such introns can be designed to have a desired strength of splice site and a desired length. In a preferred embodiment of the present invention, both the molecular switch expression cassette and the therapeutic gene expression cassette include a synthetic intron. The synthetic intron includes consensus sequences for the 5' splice site, 3' splice site, and branch point. When incorporated into eukaryotic vectors designed to express therapeutic genes, the synthetic intron will direct the splicing of RNA transcripts in a highly efficient and accurate manner, thereby minimizing cryptic splicing and maximizing production of the desired gene product.

A "therapeutic molecule" or "therapeutic gene" is one that has a pharmacologic activity when administered appropriately to a mammal suffering from a disease or condition. Such a pharmacological property is one that is expected to relate to a beneficial effect on the course or a symptom of the disease or condition. The term "therapeutic protein" as used herein refers to the native, full-length secreted form of a therapeutic protein, as well as to analogs or derivatives thereof comprising single or multiple amino acid substitutions, deletions or additions that retain native therapeutic protein function or activity. Sequences encoding therapeutic proteins may include codon-optimized versions of native sequences. Optimal codon usage in humans is indicated by codon usage frequencies for highly expressed human genes and may be determined from the program "Human High.codN" from the Wisconsin Sequence Analysis Package, Version 8.1, Genetics Computer Group, Madison, WI. The codons that are most frequently used in highly expressed human genes are presumptively the optimal codons for expression in human host cells, and thus form the basis for constructing a synthetic coding sequence.

The term "plasmid" as used herein refers to a construction comprised of extrachromosomal genetic material, usually of a circular duplex of DNA that can replicate independently of chromosomal DNA. Plasmids may be used in gene transfer as vectors.

The term "vector" as used herein refers to a construction comprised of genetic material designed to direct transformation of a targeted cell. A vector may contain

multiple genetic elements positionally and sequentially oriented with other necessary elements such that an included nucleic acid cassette can be transcribed and when necessary translated in the transfected cells. As used herein the term "expression vector" refers to a DNA plasmid that contains all of the information necessary to produce a recombinant protein in a heterologous cell.

The term "pharmacological dose" as used herein with a vector/molecular switch complex refers to a dose of vector and level of gene expression resulting from the action of the promoter on the nucleic acid cassette when introduced into the appropriate cell type that will produce sufficient protein, polypeptide, or antisense RNA to either (1) increase the level of protein production, (2) decrease or stop the production of a protein, (3) inhibit the action of a protein, (4) inhibit proliferation or accumulation of specific cell types, or (5) induce proliferation or accumulation of specific cell types. The dose will depend on the protein being expressed, the promoter, uptake and action of the protein or RNA. The term "pharmacological dose" as used herein with a ligand refers to a dose of ligand sufficient to cause either up-regulation or down-regulation of the nucleic acid cassette. Thus, there will be a sufficient level of ligand such that it will bind with the receptor in the appropriate cells in order to regulate expression from the nucleic acid cassette. The specific dose of any ligand will depend on the characteristics of the ligand entering the cell, binding to the receptor and then binding to the DNA and the amount of protein being expressed and the amount of up-regulation or down-regulation needed.

In a plasmid based expression system, a non-viral gene medicine may also be composed of a synthetic gene delivery system in addition to the nucleic acid encoding a gene product (e.g., a therapeutic protein). The non-viral gene medicine products are generally intended to have low toxicity due to the use of synthetic components for gene delivery (minimizing for instance the risks of immunogenicity generally associated with viral vectors) and non-integrating plasmids for gene expression. Since no integration of plasmid sequences into host chromosomes has been reported *in vivo* to date, they should neither activate oncogenes nor inactivate tumor suppressor genes. This built-in safety with non-viral systems contrasts with the risks associated with the use of most viral vectors. As episomal systems residing outside the chromosomes, plasmids have defined pharmacokinetics and elimination profiles, leading to a finite duration of gene expression in target tissues.

Formulating the nucleic acid with non-ionic and anionic polymers may be desirable where the polymers enhance transfection and expression of the nucleic acid or protect the nucleic acid from degradation, and are biodegradable. In addition, because formulating the nucleic acid may result in more efficient transfection, lower amounts of DNA may be used. By biodegradable, it is meant that the polymers can be metabolized or cleared by the organism *in vivo* without any or minimal toxic effects or side effects. The term "anionic polymers" means polymers having a repeating subunit that includes, for example, an ionized carboxyl, phosphate or sulfate group having a net negative charge at neutral pH. Examples of the anionic polymers include poly-amino acids (such as poly-glutamic acid, poly-aspartic acid and combinations thereof), poly nucleic acids, poly acrylic acid, poly galacturonic acid, and poly vinyl sulfate. In the case of polymeric acids, the polymer will typically be utilized as the salt form. Examples of other polymers include PVP, PVA, chitosan, etc.

Efforts have been made to enhance the delivery of plasmid DNA to cells by physical means including electroporation, sonoporation and pressure. Injection by electroporation involves the application of a pulsed electric field to create transient pores in the cellular membrane without causing permanent damage to the cell and thereby allows for the introduction of exogenous molecules. By adjusting the electrical pulse generated by an electroporetic system, nucleic acid molecules can find their way through passageways or pores in the cell that are created during the procedure. U. S. Patent No. 5,704,908 describes an electroporation apparatus for delivering molecules to cells at a selected location within a cavity in the body of a patient. Using advanced techniques of intramuscular injections of plasmid DNA followed by electroporation into skeletal muscle has been shown by the present inventors to lead to high levels of circulating GHRH. (Draghia-Akli, R., et al. (1999) *Nat.Biotechnol.* 17, 1179-1183.)

The term "pulse voltage device", or "pulse voltage injection device" as used herein relates to an apparatus that is capable of causing or causes uptake of nucleic acid molecules into the cells of an organism by emitting a localized pulse of electricity to the cells, thereby causing the cell membrane to destabilize and result in the formation of passageways or pores in the cell membrane. It is understood that conventional devices of this type are calibrated to allow one of ordinary skill in the art to select and/or adjust the desired voltage amplitude and/or the duration of pulsed voltage and therefore it is expected that future devices that perform this function will also be calibrated in the same manner.

The type of injection device is not considered a limiting aspect of the present invention. The primary importance of a pulse voltage device is, in fact, the capability of the device to facilitate delivery of compositions of the invention into the cells of an organism. The pulse voltage injection device can include, for example, an electroporetic apparatus as described in U.S. Patent 5,439,440, U.S. Patent 5,704,908 or U.S. Patent 5,702,384 or as published in PCT WO 96/12520, PCT WO 96/12006, PCT WO 95/19805, and PCT WO 97/07826, all of which are incorporated herein by reference in their entirety.

EXAMPLE 1:

In one embodiment of the present invention, an early-regulated expression system was tested for GHRH expression. In a first embodiment, the inducer plasmid was pGLV65, which codes for an early version of the regulator protein, and contains a synthetic muscle specific promoter SPc5-12 promoter driving expression of an early version chimeric regulator protein comprised of a GAL-4 1-94aa DNA binding domain, a modified progesterone receptor ligand binding domain and a NFκBp65 transactivator domain (SEQ.ID.NO. 12) followed by a SV40 poly(A) signal. The early regulated plasmid, p1450HV-GHRH (encoding protease resistant super-porcine GHRH), was similar to the present improved regulated plasmid with the exception that it includes the INR region downstream of the TATA that was deleted in the improved inducible plasmid, pGHRH1674.

The inducible system was co-delivered by intra-muscular injection to SCID mice in a total quantity of 30 micrograms. At ten days post-injection, RU486, was injected i.p. at a dose of 250 micrograms/kg for 3 days. On the fourth day, the animals were bled and serum was used to measure IGF-I levels. Upon repeated administration of RU486 to the animals using the same protocol (three days induction, bleed the fourth day, allow recovery to background 7 days) over 90 days, serum IGF-I levels rose repeatedly 1.05-1.23 fold over the uninjected controls.Although no organomegaly or associated pathology was observed in the injected animals, weight and body composition in SCID mice injected with the early inducible system was unaltered upon pulse activation of the system. A slight increase in the lean to total body mass is observed in animals receiving the constitutively active construct. Furthermore, in this system, the ability to regulate expression was eventually compromised by an increase in apparent basal expression. As shown in Figure 14, after the second induction, IGF-I levels did not return to baseline and

eventually increased in the absence of inducer drug. In further embodiments, a system having tightened regulation of transgene expression in the absence of inducer was developed.

EXAMPLE 2: ***Modifications to the Transcribed but Untranslated Portions of the GENESWITCH® Plasmid***

In one embodiment of the present invention, the tightness of regulated expression was improved by modifying certain aspects of the GENESWITCH® and inducible plasmids. Modifications, as will be discussed in succeeding examples included: modifications to the transcribed but untranslated portions of the GENESWITCH® plasmid and truncation of the GAL-4 DNA binding domain. Modification of the core promoter on the inducible therapeutic gene plasmid was also undertaken in the improved system to reduce basal expression while retaining high inducability. It should be noted that these modifications may be applied independently or in combination with the improved GENESWITCH® protein modifications.

The transcribed portions of the GENESWITCH® plasmid were modified to include post-transcriptional elements (5' UTR including a synthetic intron and poly (A) signal) that could be expected to improve the level and fidelity of transgene expression. In a preferred embodiment of the present invention, both the molecular switch expression cassette and the therapeutic gene expression cassette include a synthetic intron.

Cryptic splicing in transcripts from eukaryotic expression vectors is obviously undesirable. To obtain control over the splicing pattern and to maximize gene expression, suboptimal introns can be replaced by a strong intron. A synthetic intron with consensus splicing sequences should be optimal for this purpose. The synthetic intron of the present embodiment includes consensus sequences for the 5' splice site, 3' splice site and branch point. When incorporated into eukaryotic vectors designed to express therapeutic genes, the synthetic intron will direct the splicing of RNA transcripts in a highly efficient and accurate manner, thereby minimizing cryptic splicing and maximizing production of the desired gene product.

The first and sixth position of the 5' splice site consensus sequence are partially ambiguous. The 5' splice site pairs with U1 snRNA. The chosen sequence minimizes the free energy of helix formation between U1 RNA and the synthetic 5' splice site.

```
5'ss        5' CAGGUAAGU 3'   SEQ.ID.NO: 1
                |||||||||
U1 RNA      3' GUCCAUUCA 5'   SEQ.ID.NO: 2
```

In mammals, the branch point sequence is very ambiguous. The branch point sequence, except for a single bulged A residue, pairs with U2 snRNA. The chosen sequence minimizes the free energy of helix formation between U2 RNA and the synthetic branch point sequence. It also matches the branch point sequence that is obligatory for yeast pre-mRNA splicing. The branch point is typically located 18-38 nts upstream of the 3' splice site. The branch point of the synthetic intron is located 24 nts upstream from the 3' splice site.

```
BP          5' UACUA^A C 3'   SEQ.ID.NO: 3
                |||||  |
U2 RNA      3' AUGAU G 5'     SEQ.ID.NO: 4
```

The polypyrimidine tract of the consensus sequence for 3' splice sites is not exactly defined. At least 5 consecutive uracil residues are needed for optimal 3' splice site function. This concept is incorporated into the polypyrimidine tract of the synthetic intron, which has 7 consecutive uracil residues.

Splicing *in vitro* is optimal when introns are >80 nts in length. Although many introns may be thousands of bases in length, most naturally occurring introns are 90-200 nt in length. The elemental structure of a synthetic intron according to the present invention (SEQ.ID.NO:5) is shown in Figure 3 compared with italicized consensus sequences (SEQ.ID. NO:6).

In one synthetic intron embodiment of the present invention, IVS8, the length of the synthetic intron is 118 nucleotides. The sequence of IVS8, (SEQ.ID.NO:7), is shown in Figure 4. Exonic sequences are in boldface. N = any base. Consensus splicing signals are double-underlined. Restriction enzyme recognition sites are over-lined. The restriction enzyme BbsI may be used to cleave the DNA precisely at the 5' splice site, and EarI may be used to cleave the DNA precisely at the 3' splice site. The two restriction sites, BbsI and EarI, located within the synthetic intron, permit the intron to be easily and precisely deleted. The PstI and NheI sites are included to facilitate the verification of

cloning procedures. Double-stranded DNA with this sequence may be prepared using mutually priming long oligonucleotides.

To more closely match the structure of naturally occurring genes, which typically contain many introns, the synthetic intron may be inserted into the gene of interest at multiple locations. When multiple introns are inserted, however, care must be taken to ensure that the lengths of resultant internal exons are less than 300 nucleotides. If internal exons are greater than 300 nucleotides in length, exon skipping may occur.

In one embodiment, the expression cassette was further modified to introduce a CMV 5' UTR, termed UT12 (SEQ. ID. NO:8) in addition to the synthetic intron, IVS8 (SEQ.ID.NO:7), within the 5' UTR. The SV40 polyadenylation signal was replaced with a human growth hormone ("hGH") poly (A) signal.

These modifications, or other expression cassette modifications known to those of skill in the art, may be employed to generally increase the level and fidelity of transgene expression from plasmid and viral vectors.

EXAMPLE 3: ***Truncation of the GAL-4 DNA Binding Domain***

The GAL-4 DNA binding domain binds as a dimer to the palindromic 17-mer GAL-4 DNA binding site (CGGAAGACTCTCCTCCG, SEQ.ID.NO.9). The Kd for binding of GAL-4, residues 1-100, is 3 nM (Reece and Ptashne (1993) *Science* 261: 909-911). Thus, in order to bind to the GAL-4 promoter and activate transcription of the inducible GHRH plasmid, it is contemplated that a GENESWITCH® regulatory protein having a GAL-4 DNA binding domain is required to form a homodimer. In the presence of the inducer, MFP, binding of MFP to the mutated hPR LBD may trigger a conformational change in the protein so as to initiate dimerization.

As discussed in Example 1, however, increases in IGF-I levels were observed in the presence of the pGLV65 protein even without the MFP ligand, indicating that the early version of the GAL-4 ligand binding domain protein comprising aa 1-93 may be able to dimerize and bind the GAL-4 binding site and induce transgene expression in the absence of MFP. Efforts were undertaken to reduce this drug-independent induction of expression by considering the tertiary structure of GAL-4 using molecular modeling and designing truncation mutant having lower dimerization potential but retaining sequence-specific DNA binding activity.

Figure 5 depicts the structure of the GAL-4 protein DNA binding domain, residues 1-93 SEQ. ID. NO. 10; residues 2-93 of which has been incorporated into the early version molecular switch plasmids (correlating to the underlined nucleic acid sequence in Figure 7, SEQ. ID. NO. 12, and amino acid sequence in Figure 8, SEQ. ID. NO. 14). The DNA recognition unit (residues 9-40) is boxed, with the cysteine (C) residues involved in chelating zinc shown in bold. The coiled-coil structures that form the dimerization elements (residues 54-74 and 86-94) are also boxed, with the generally hydrophobic first and fourth positions of each heptad repeat sequence shown in bold. Residue Ser 47 and Arg 51, which form an H-bond between chains, are marked by carats.

The first seven residues of the GAL-4 DNA binding domain are disordered and are not known to contribute any function, while residues 8-40 form the Zn binding domain or the DNA recognition unit. This unit has two alpha helical domains that form a compact globular structure and in the presence of Zn resulting in a structure that is a binuclear metal ion cluster rather than a zinc finger, i.e., the cysteine-rich amino-acid sequence (Cys^{11}-Xaa_2-Cys^{14}-Xaa_6-Cys^{21}-Xaa_6-Cys^{28}-Xaa_2-Cys^{31}-Xaa_6-Cys^{38}, SEQ.ID.NO.11) binds two Zn(II) ions (Pan and Coleman (1990) *PNAS* 87: 2077-81). The Zn cluster is responsible for making contact with the major groove of the 3 bp at extreme ends of the 17-mer binding site. A proline at 26 (cis proline) forms the loop that joins the two alpha-helical domains of the zinc cluster domain and is also critical for this function.

Residues 41-49 exist as a disordered linker that joins the DNA recognition unit and the dimerization elements (54-74 and 86-94). Once dimerized, residues 47-51 of dimerized subunits also interact with phosphates of the DNA target. Residues 50-64 are contemplated to be involved in weak dimerization. They consist of a short coiled-coil that forms an amphipathic alpha-helix and wherein two alpha-helices are packed into a parallel coiled-coil similar to a leucine zipper. In addition to hydrophobic interactions of 3 pairs of leucines and a pair of valines found within residues 54-74, there are two pairs of arg-glu salt links, and H-bonds between Arg 51 of one monomer to Ser 47 of the other monomer. Residues 65-94 are contemplated to form a strong dimerization domain. The structure of residues 65-71 has not been fully determined, but it is most likely a continuation of the coiled-coil structure for one heptad repeat. Residues 72-78 contain a proline and therefore disrupt the amphipathic helix. Residues 79-99, however, contain three more potentially alpha-helical heptad sequences (Marmorstein et al (1992) *Nature* 356: 408-414).

There are a number of possible modifications that can be made to the regions of the GAL-4 domain as discussed above. Modifications in these regions may result in lower basal expression but still retain sequence-specific DNA binding. For example, the length of the region that contains the interacting coiled-coil sequences (residues 54-74 and residues 86-93) could be shortened by deletion such as deleting amino acid sequence 54-64, 65-74, 54-74, or 86-93. As such, GAL-4 mutants with only one coiled-coil region could be constructed by deleting one of the coiled-coil region. In addition, mutant or artificial sequences may also be used to replace the fragment GAL-4 domain with unique restriction sites positioned at key spots, for example at the junctions of each of the alpha-helical heptad sequences. Thus, modified versions of the GAL-4 protein domain could be produced that have progressively reduced alpha-helical heptad sequences.

In one exemplary embodiment of the present invention, the GAL-4 domain was truncated by deletion of amino acids 75-93. This was achieved through the use of a convenient restriction endonuclease sites (Hinc II and Xho I) although other truncations may be produced according to molecular biology techniques known to those of skill in the art. By deleting the 72 bp XhoI-HincII fragment, GENESWITCH® v.4.0, embodied in the pGS1633, was generated, which has a 19 amino acid truncation at the C-terminal portion of the GAL-4 DNA-binding domain (the deletion corresponding to amino acid sequence 75-93 of SEQ. ID. NO. 10 of in Figure 5). Figure 7 shows the nucleic acid sequences of the coding region of both early version, pGLV65, SEQ. ID. NO. 12, and an improved GENESWITCH®v.4.0, embodied in pGS1633, SEQ. ID. NO. 13, while Figure 8 shows the respective amino acid sequences of the same, SEQ. ID. NOS. 14 & 15. Molecular modeling indicates that deletion of residues 75-93 removed the C-terminal helical portion without affecting the helical and coiled structure of the upstream amino acid sequences. As is apparent in Figure 7, the N-terminal methionine of the native GAL-4 sequence has been removed and a further eight amino acids have been added to the N-terminal end of the GENESWITCH® protein. Modifications to the N-terminal seven amino acids region are not of consequence as long as they do not affect the tertiary structure of residues 8-40 of the Zn binding domain.

It is notable that a 1-74 amino acid GAL-4 domain has been reported in a regulator protein chimera having a native estrogen-receptor ligand-binding domain and a VP-16 transactivation domain. Webster, J. et al., *Cell* 54:199 (1988). However, this construct was reported to have only 50% of the activity of a construct having amino acids 1 –147 of

the GAL-4 domain. In contrast, the present construct having GAL-4 (2-74) together with a mutated progesterone receptor ligand-binding domain was as active as a GAL-4 1-93 construct in the presence of ligand while having lower background in the absence of ligand.

EXAMPLE 4: ***Amino Acid Sequence of the GeneSwitch Protein***

In another embodiment of the present invention, the improved molecular switch protein may have the amino acid sequence: MX_n/ [yGAL-4 $_{2-74}$] / X_n / [hPR $_{640-914}$] / X_n / [hP65 $_{285-551}$] as exemplified by SEQ.ID.NO:16 wherein X_n represents a series of amino acid sequences. X can be any amino acid and n can be any number but preferably between 1-10. In another embodiment of the present invention, the improved molecular switch protein, encoded for example in GS v4.0 has the amino acid sequence MDSQQPDL / [yGAL-4 $_{2-74}$] / DQ / [hPR $_{640-914}$] / GST / [hP65 $_{285-551}$] as exemplified by (SEQ.ID.NO:17). Amino acids for the N-terminus and linker regions are indicated by single letter abbreviations. Individual protein components are bracketed and the amino acid residues are specified in subscript. yGAL-4 is the N-terminal DNA binding domain of the yeast GAL-4 protein (GenBank accession no. AAA34626). hPR is a C-terminal truncated portion of the ligand binding domain of the human progesterone receptor (GenBank accession no. AAA60081), where $hPR_{640-933}$ is the full-length version of the hPR-LBD. hP65 is the activation domain of the p65 subunit of human NF-κB (GenBank accession no. AAA46408). The human components of the GENESWITCH® regulator protein comprise 86% of its sequence.

A map of an exemplary regulated GHRH plasmid, pGHRH1674 is shown in Figure 10 with the corresponding sequence, SEQ.ID.NO:29, on Figure 12 (GHRH sequence indicated).

EXAMPLE 5: ***Modification of the Transgene Core Promoter***

The core region of the inducible promoter in the regulated GHRH plasmid was also modified to reduce the basal expression of GHRH without reducing the inducibility of GHRH expression. This was desirable because, as shown in Figure 14 increases in IGF-I levels were ultimately observed in the absence of MFP when the original inducer and inducible GHRH plasmids were delivered (in the absence of GENESWITCH® plasmid) to mice. Studies conduced with an analogous inducible plasmid showed that a certain level of basal expression was obtained in the absence of the regulator plasmid indicating a

certain level of basal expression from the single inducible plasmid in the absence of the regulator protein. It was, thus, desirable to develop a system in which the basal expression from the regulated plasmid was significantly reduced in order to increase reliance on administration of the inducer drug and provide an increased margin of safety by virtue of extrinsically controlled expression rather than through dependence on plasmid administration dose.

In one embodiment of the present invention, the promoter of an inducible GHRH plasmid contains 6X GAL-4 sites linked to a TATA box. (Figure 6B) Different numbers of GAL-4 sites may be employed and the optimal number may be determined empirically. Figure 6B depicts the nucleotide sequence of an inducible 6X GAL-4/TATA promoter (SEQ.ID.NO. 18). The six GAL-4 elements (17 bp in length) are boxed, the TATA box (-29 to –24) is double-underlined, and the predicted transcription initiation site (+1) is marked by the arrow. The sequence from –33 to –22, which contains the TATA box, is from the E1b region of Adenovirus type 2 (residues 1665-1677 of NCBI accession no. J01917).

Utilizing unique restriction endonuclease sites engineered into the core promoter region, different regions of the promoter and 5' UTR were deleted to determine what effect, if any, this would have on overall expression of the transgene in the presence or absence of the GENESWITCH® protein. Figure 6A indicates schematically the unique restriction endonuclease sites used to delete the TATA box (Sal I/EcoR V), inr (EcoR V/BsmB I) and part of the UT12 (Sac II/Pac I). Figure 6C depicts the sequence of the inducible promoter, SEQ. ID. NO. 19, with relevant regions and unique restriction enzyme sites boxed. BsmBI enzyme cleavage sites are indicated with arrows. "TF" refers to transcription factor binding site, and "inr" refers to initiator.

To reduce the basal activity of the inducible promoter, an "inr" region as depicted in encompassed in a 30 bp BsmBI-EcoRV fragment (13-42 bp downstream from the TATATAAT box) was deleted as shown in Figure 6C. It was found that resultant inducible plasmids having a deletion in the inr region, the level of transgene expression was 7 - 10X lower than with inducible promoter having the inr in the absence of a GENESWITCH® plasmid.

On the other hand, when the plasmids lacking the inr were co-transfected with a GENESWITCH plasmid, the inducibility of expression was increased in some cases from 5X inducibility to 7X inducibility. Hence, a deletion in the transcription initiation region

(inr) of the inducible plasmid significantly reduces the intrinsic activity of the promoter without impairing, and may even enhance, its ability to be induced.

EXAMPLE 6: ***Muscle Specific Promoter in the GENESWITCH® Plasmid***

Where expression in a particular tissue is desired, strong non-tissue specific promoters may be replaced with tissue specific promoters. For example, if the target tissue for gene expression is muscle, an actin promoter may be employed. Several advantages may be gained through the use of tissue-specific promoters. In a particular tissue, such as for example, muscle tissue, use of muscle-specific promoters may increase the fidelity of expression. Tissue-specific promoters may be expected to decrease the potential for occult gene expression in non-target tissues. In particular, tissue-specific promoters may provide the advantage of reduced expression in dendritic and other antigen presenting cells, thus avoiding immune responses to the expressed proteins. In certain circumstances, a low level of regulator plasmid expression may also be desirable. In a combination plasmid system, it is also preferable to regulate the level of transgene expression by inherent properties of the plasmid delivered rather than by attempting to variably titrate the dose of plasmid delivered.

In one embodiment, the promoter of the GENESWITCH® plasmid was a muscle-specific promoter, avian skeletal α-actin promoter (SK promoter) (SEQ. ID. NO. 20) to reduce the level of regulator protein produced. The avian skeletal α-actin promoter is described in US Patent No. 5,298,422, incorporated herein by reference in its entirety.

In another embodiment, the muscle specific promoter is a synthetic muscle specific promoter comprised of a series of muscle specific transcriptional regulatory regions having a novel configuration relative to those found in nature as described in WO9902737, incorporated herein by reference in its entirety. In addition, in one aspect of the present invention a unique synthetic promoter is utilized, termed SPc5-12 (Li et al., (1999), *Nature Biotechnology*), which contains a proximal serum response element (SRE) from skeletal α-actin, multiple MEF-2 sites, MEF-1 sites, and TEF-1 binding sites, and exceeds the transcriptional potencies of natural myogenic promoters. Examples of synthetic muscle specific promoters include SPc 5-12, SEQ.ID.NO. 22 and SPc1-28, SEQ.ID.NO. 21, comprising various synthetic orientations and combinations of muscle specific transcriptional regulatory regions including SRE, MEF-1, MEF-2, TEF-1 and SP-1, the sequences of which are set out below with the critical sequences underlined.

SRE	5'----GACACCCAAATATGGCGACGG----3'	21 mer	SEQ.ID.NO. 23
MEF-1	5'----CCAACACCTGCTGCCTGCC----3'	19 mer	SEQ.ID.NO. 24
MEF-2	5'----CGCTCTAAAAATAACTCCC----3'	19 mer	SEQ.ID.NO. 25
TEF-1	5'----CACCATTCCTCAC----3'	13 mer	SEQ.ID.NO. 26
SP1	5'----CCGTCCGCCCTCGG----3'	14 mer	SEQ.ID.NO. 27

In another embodiment, the muscle-specific avian skeletal α-actin promoter was combined with an optimized arrangement of post-transcriptional elements (5' untranslated region (UT12), synthetic intron (ivs 8) and poly(A) signal (hGH pA)).

As depicted in Figure 1, the improved plasmid (pGS1633) codes for GENESWITCH® regulator protein v.4.0 constructed by deleting about 20 C-terminal residues of the GAL-4 region (indicated by the inverted triangle in Figure 1) together with a muscle-specific promoter.

EXAMPLE 7: ***Expression of GHRH in a Tightly Regulated Expression System In Vitro***

Minimal Essential Medium (MEM), heat-inactivated horse serum (HIHS), gentamycin, Hanks Balanced Salt Solution (HBSS), and lipofectamine were obtained from Gibco BRL (Grand Island, NY). Primary chicken myoblast cultures were obtained and transfected as described in Bergsma, D. J., et al. (1986) *Molecular & Cellular Biology* 6, 2462-2475 and Draghia-Akli, R., et al. (1997) *Nature biotechnology* 15, 1285-1289.

A 228-bp fragment of super-porcine GHRH, HV-GHRH (Draghia-Akli, R., et al. (1999) *Nat.Biotechnol.* 17, 1179-1183), followed by the 3' untranslated region of the human GH (hGH) cDNA was incorporated into inducible GHRH expression vectors. Primary myoblasts were transfected with a mixture of 1:10 GeneSwitch/ inducible GHRH plasmid, pGS1633/pGHRH1674 (Inducer System "IS"). After transfection, the medium was changed to MEM which contained 2% HIHS to allow the cells to differentiate. At 24 and 48 hours after transfection, cells were washed in PBS, and mifepristone (Biomol Research Laboratories, Plymouth Meeting, PA), diluted in culture media from a stock solution of 0.01 M in ethanol, was added. Media and cells were harvested 72 hours post-differentiation. Non-induced controls were transfected, but MFP was not added to the media.

For Northern analysis of extracted total RNA, 20 micrograms of total RNA was DNase I treated (Gibco BRL), size separated in 1.5% agarose-formaldehyde gel and transferred to Gene Screen nylon membrane (DuPont Research Products, Boston, MA). The membranes were hybridized with a GHRH cDNA probe ^{32}P-labeled by random priming (Ready-to-Go DNA labeling kit, Pharmacia Biotech, Piscataway, NJ). Hybridization was carried out at 45°C in a solution which contained 50% formamide, 5xSSPE, 5xDenhardt's, 1% SDS, 200 microliters/ml sheared salmon sperm DNA. Membranes were washed twice for 10 minutes in 2xSSPE/1%SDS at room temperature and twice for 30 minutes in 0.2xSSPE/1%SDS at 68°C. Blots were subsequently exposed to X-ray film (Kodak X-Omat AR; Eastman Kodak, Rochester, NY) at -80°C with intensifying screens.

The IS activity was compared with that of a positive control, a constitutively active construct (SP-GHRH). As shown in Figure 15, at the end of the experiment specific GHRH mRNA profiles were analyzed by Northern blotting, using a GHRH specific probe. Northern blot analysis in which an equal quantity of mRNA was loaded on each lane, showed the expected size transcripts of 0.35kb only in myoblasts transfected with the IS in the presence of MFP, and in the positive control.

EXAMPLE 8: ***Expression of GHRH in a Tightly Regulated Expression System In vivo***

Intramuscular injection of plasmid DNA in adult mice. Severe combined immunodeficient (SCID) male mice (Taconic Laboratories, Germantown, NY) were housed and cared for in the animal facility of Baylor College of Medicine (Houston,TX). Animals were maintained under environmental conditions of 10h light/14h darkness, in accordance with NIH Guide, USDA and Animal Welfare Act guidelines, and a protocol approved by the Institutional Animal Care and Use Committee. On day 0, the animals (n = 20 group) were weighed and then the left tibialis anterior muscle of mice was injected with 10 micrograms of the inducible system (IS) (1:10 transactivator to target gene), in 25 microliters PBS. The injection was followed by caliper electroporation, as described in Draghia-Akli, R., et al. (1999) *Nat.Biotechnol.* 17, 1179-1183.

At twenty-one days post-injection, MFP at 250 micrograms/kg was injecting i.p. for 3 days. On the fourth day, the animals were bled and serum was used to measure IGF-I levels. Repeated administration of MFP to the animals using the same protocol (three days induction, bleed the fourth day, allow recovery to background 7 days) was performed

four times over 115 days. In between day 124 and 149, MFP was administered daily. At the end of the experiment, body composition was performed *in vivo*, using a dual x-ray absorbimetry technique, PIXImus, and than post-mortem. Blood was collected, centrifuged immediately at 0°C, and stored at -80°C prior to analysis. Organs, carcass, fat from injected animals and controls were removed, weighed and snap frozen in liquid nitrogen.

Body composition data: Body composition measurements were performed either under anesthesia, at day 149 post-injection (PIXImus) or post-mortem (organ, carcass, body fat, direct dissection followed by neutron activation analysis of the body).

Statistics: Values shown in the figures are the mean ± s.e.m. Specific p values were obtained by comparison using Students t-test or ANOVA analysis. A $p<0.05$ was set as the level of statistical significance.

DNA constructs. Plasmid pGS1633, encoding improved GeneSwitch regulator protein having a truncated GAL-4 DNA binding domain under the control of a muscle specific skeletal alpha-actin promoter, was used together with an improved regulated plasmid, pGHRH1674, encoding a mutated GHRH cDNAs obtained by site-directed mutagenesis of human GHRH cDNA (Altered Sites II *in vitro* Mutagenesis System, Promega, Madison, WI). The GHRH cDNA is followed by the 3' untranslated region of human GH hormone.

Administration and Results: On day 0, the animals were weighed and then, the left tibialis anterior muscle of mice was injected with 10 micrograms of the inducible system (IS) (1:10 transactivator to target gene), in 25 microliters PBS. The injection was followed by caliper electroporation. At twenty-one days post-injection, MFP was injected i.p., 250 micrograms/kg for 3 days. On the fourth day, the animals were bled and serum was used to measure IGF-I levels. Upon administration of MFP for 4 consecutive days, up-regulation of IGF-I levels was observed (1797.28 ± 164.96 ng/ml versus 1100.86 ± 33.67 ng/ml pre MFP levels in the same group of animals, $p < 0.0006$, 1086.78 ± 65.34 ng/ml in control (beta-galactosidase) injected animals, $p < 0.0007$, 1171.79 ± 42 ng/ml in animals injected with the two plasmids, but did not receive the inducer drug, $p < 0.002$, and animals injected with the constitutively active construct 1374.22 ± 83.8 ng/ml, $p < 0.03$). Upon repeated administration of MFP to the animals using the same protocol (three days induction, bleed the fourth day, allow recovery to background 7 days) over 110 days, serum IGF-I levels rose repeatedly 1.1-1.7 fold over the uninjected controls (Figure 16).

Body and organ weights in injected mice. Body weight was similar in between groups for the first 124 days of the study (Figure 17 A). In between day 124 and 149, the mice were MFP-induced every day. Body weight increased by 7.5 % in chronically MFP-induced GHRH/GeneSwitch animals, that averaged 31.84 ± 0.12g ($p < 0.027$), compared with b-gal controls 29.62 ± 0.98g, and no MFP induced animals that averaged 30.53 ± 0.59g. At the end of the experiment, body composition was analyzed *in vivo*, by DEXA, using a high resolution PIXImus scanner, and subsequently post-mortem. Organs (lungs, heart, liver, kidney, stomach, intestine, adrenals, gonads, brain) were collected and weighted. No organomegaly or associated pathology was observed in the injected animals. Pituitary glands were dissected within the first minutes post-mortem, and weighted. Pituitary weight / total body weight (Figure 17B) increased upon chronic stimulation of the GHRH/GeneSwitch by 20% ($7.35 \pm 0.31 \times 10^{-5}$), compared with β-gal controls ($6.13 \pm 0.46 \times 10^{-5}$), and no MFP animals ($6.3 \pm 0.22 \times 10^{-5}$), $p < 0.035$. There is no significantly statistical difference in between the β-gal injected animals and animals that were injected with the GHRH/GeneSwitch system, but not given MFP. Our hypothesis is that the increase in pituitary weight is due to somatotrophs hypertrophy, as it is known that GHRH is stimulating synthesis and secretion of GH from the anterior pituitary, and has a specific hypertrophic effect on somatotrophs. In conjunction with GHRH, normal GHRH receptors and pit-1, as present in our animals, are necessary for functional changes within the pituitary.

Body composition in activated GHRH/GeneSwitch animals. Body composition studies by dual-energy x-ray absorptiometry, PIXImus (total body fat, non-bone lean tissue mass and bone mineral area, content and density) showed significant changes in chronically MFP induced animals injected with the GHRH/GeneSwitch system. Lean, non-bone body mass (Figure 18 A) increased by 2.5% in GHRH/GeneSwitch animals + MFP (87.44 ± 0.65%, versus β-gal 84.94 ± 0.6%, and no MFP animals 84.88 ± 0.3%), $p < 0.022$. Fat mass (Figure 18 B) decreased by 2% in GHRH/GeneSwitch animals (12.59 ± 0.62%, versus β-gal 14.57 ± 0.75%, and no MFP animals 15.09 ± 0.3%), $p < 0.05$.

Upon chronic stimulation of the GHRH/ GeneSwitch system, significant changes occurred in bone area (Figure 19A), that increased by 7 %, (12.81 ± 0.14 cm^2, versus β-gal injected controls 11.98 ± 0.3 cm^2, or no MFP animals 12.07 ± 0.26 cm^2), $p < 0.0006$, bone mineral content (Figure 19B) increased by 14.6% (0.755 ± 0.012g, versus β-gal injected

controls 0.659 ± 0.019g, or no MFP animals 0.694 ± 0.023 cm^2), $p < 0.002$, and bone mineral density increased by 6% (0.059 ± 0.0007 g/cm^2, versus β-gal injected controls 0.056 ± 0.0009 g/cm^2, or no MFP animals 0.057 ± 0.0007 g/cm^2), $p < 0.012$. Practically, there is no overall difference in between the β-gal injected animals and animals that were injected with the GHRH/GeneSwitch, but not given MFP, proving a tight regulation of the system.

Treated animals did not experience any side effects from the therapy, had normal biochemical profiles, and with no associated pathology or organomegaly. The profound increases in IGF-I levels, enhancement in growth and changes in body composition, upon chronic induction of the GHRH/Gene Switch system indicate that ectopic expression of myogenic GHRH vectors has the potential to replace classical GH therapy regimens and may stimulate the GH axis in a more physiologically appropriate manner.

EXAMPLE 9: ***Pulsatile and Diurnal Control of GHRH Expression***

Growth hormone is released in a pulsatile or episodic fashion and observes a diurnal pattern of increased noctural release. Growth hormone is released within 20 minutes of infusion of recombinant GHRH. GHRH has a very short half-life. In i.v. studies in children, GHRH 1-40, 1 micrograms/kg, demonstrated a distribution half life (t1/2) of 3.9 (SD 0.9) min and an elimination t1/2 of 53.1 (SD 3.2) min. Smith PJ, et al. (1987) *Clin Endocrinol (Oxf)* Oct;27(4):501-7.

Peak growth hormone release following continuous GHRH infusion has been reported to be similar to that of maximally effective bolus doses. However, with continuous infusions, the GH response is not sustained; and immediately after GHRH infusions, the response to previously effective bolus doses is reduced possibly reflecting either receptor-mediated desensitization, the depletion of rapidly releasable GH stores, or both. Gelato MC. et al. (1985) *J Clin Endocrinol Metab* 61(2):223-8. In a study comparing the effect of continuous infusion of GHRH versus daily pulsatile administration on GH release in lambs, it was found that basal plasma GH levels were increased after chronic pulsatile GHRH treatment but not after any kind of continuous GHRH administration. Perez-Romero A, et al. (2000) *J Physiol Biochem* 56(2):107-15.

Thus, in one embodiment, the present invention provides a method of pulsatile exposure to GHRH after a single administration of DNA encoding GHRH in which

GHRH is produced in vivo following pulsatile administration of mifepristone resulting in activation of the GeneSwitch protein followed by expression of GHRH.

In another embodiment, the present invention provides a method of diurnal exposure to GHRH after a single administration of DNA encoding GHRH in which GHRH is produced in vivo following diurnal administration of mifepristone resulting in activation of the GeneSwitch protein followed by expression of GHRH.

All patents and publications are hereby incorporated herein by reference as if they are fully set forth herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. Transformed cells, vectors, compositions, molecular switches and receptors, along with the methods, procedures, treatments and molecules described herein are exemplary and representative of preferred embodiments. They are not intended as limitations on the scope of the invention. Hence, changes to and combinations of the examples describe herein are encompassed within the spirit of the invention as defined by the scope of the claims.

WE CLAIM:

1. An inducible GHRH expression system comprising:

a first expression cassette having a nucleic acid sequence encoding a ligand specific molecular switch protein, wherein the molecular switch is inactive in the absence of the ligand; and

a second expression cassette encoding a promoter and a gene coding for a protein having a GHRH activity sufficient to induce production of IGF-I *in vivo*, wherein expression from the gene is induced by binding of the molecular switch protein to the promoter and wherein a level of protein sufficient to induce production of IGF-I is not observed in the absence of ligand.

2. The inducible GHRH expression system of claim 1 wherein the ligand specific molecular switch protein comprises:

a mutated GAL-4 DNA-binding domain;

a transregulatory domain; and

a mutated ligand-binding domain of a steroid-hormone receptor.

3. The inducible expression system of claim 2 wherein the mutated GAL-4 DNA binding domain carries a mutation in a helical domain located in the region from amino acid 54 – 93 of SEQ.ID.NO:10.

4. The inducible expression system of claim 3 wherein the mutation is selected from a group consisting of a deletion, substitution, or addition.

5. The inducible expression system of claim 4 wherein the mutation is a deletion of the amino acid 75-93 of SEQ. ID. NO. 10.

6. The inducible expression system of claim 5 wherein the mutated GAL-4 DNA binding domain consists of amino acid sequence 2-74 of SEQ. ID. NO. 10.

7. The inducible expression system of claim 4 wherein the mutation is a deletion of amino acids 54-74 of SEQ. ID. NO. 10.

8. The inducible expression system of claim 4 wherein the mutation is a deletion of amino acids 54-64 of SEQ. ID. NO. 10.

9. The inducible expression system of claim 4 wherein the mutation is a deletion of amino acids 65-74 of SEQ. ID. NO. 10.

10. The inducible expression system of claim 4 wherein the mutation is a deletion of amino acids 86-93 of SEQ. ID. NO. 10.

11. The inducible expression system of claim 1 wherein the first expression cassette further comprises a tissue-specific promoter.

12. The inducible expression system of claim 11 wherein the tissue-specific promoter is a muscle-specific promoter.

13. The inducible expression system of claim 12 wherein the muscle-specific promoter is selected from the group consisting of an α-actin promoter and a synthetic muscle specific promoter.

14. The inducible expression system of claim 1 wherein the first expression cassette further comprises a 5' untranslated region, a synthetic intron, and a poly (A) signal sequence.

15. The inducible expression system of claim 14 wherein the 5' untranslated region comprises SEQ. ID. NO. 8.

16. The inducible expression system of claim 14 wherein the synthetic intron comprises a sequence selected from a group consisting of SEQ. ID. NO. 5 and SEQ. ID. NO. 7.

17. The inducible expression system of claim 14 wherein the poly (A) signal is derived from the poly (A) signal of human growth hormone.

18. The inducible expression system of claim 2 wherein the transregulatory domain is selected from a group consisting of NFκBp65, VP-16, TAF-1, TAF-2, TAU-1, and TAU-2.

19. The inducible expression system of claim 2 wherein the steroid-hormone receptor is a progesterone receptor.

20. The inducible expression system of clause 19 wherein the mutated ligand-binding domain of the progesterone receptor carries a mutation within amino acids 813 - 933 of the human progesterone receptor.

21. The inducible expression system of clause 19 wherein the mutation is a deletion of a portion of the ligand-binding domain of the human progesterone receptor (hPR), and wherein the portion is selected from a group consisting of amino acids 813-933 of the hPR, amino acids 873-933 of the hPR, amino acids 891-933 of the hPR, and amino acids 914-933 of hPR.

22. The inducible expression system of claim 2 wherein the molecule switch protein comprises an amino acid sequence selected from SEQ. ID. NO. 14 and SEQ. ID. NO. 15.

23. The inducible expression system of claim 1, wherein the ligand is a synthetic or non-natural ligand.

24. The inducible expression system of claim 2 wherein the ligand binds to the mutated ligand-binding domain and causes the molecular switch protein to form a homodimer.

25. The inducible expression system of claim 24 wherein the ligand is selected from a group consisting of 11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-propinyl-4, 9-estradiene-3-one (RU38486 or mifepristone); 11β-(4-dimethylaminophenyl)-17α-hydroxy-17β-(3-hydroxypropyl)-13 α methyl-4, 9-gonadiene-3-one (ZK98299 or Onapristone); 11β-(4-acetylphenyl)-17β-hydroxy-17α-(1-propinyl)-4,9-estradiene-3-one (ZK112993); 11β-(4-dimethylaminophenyl)-17β-hydroxy-17α-(3-hydroxy-1(Z)-propenyl-estra-4,9-diene-3-one (ZK98734); (7β,11β,17β)-11-(4-dimethylaminophenyl)-7-methyl-4',5'-dihydrospiro [ester-4,9-diene-17, 2' (3'H)-furan]-3-one (Org31806); (11β,14β,17α)-4',5'-dihydro-11-(4-dimethylaminophenyl)-[spiroestra-4, 9-diene-17,2'(3'H)-furan]-3-one (Org31376); 5-α-pregnane-3,2-dione.

26. The inducible expression system of claim 2 wherein the promoter region of the gene comprises a GAL-4 binding site.

27. The inducible expression system of claim 26 wherein the GAL-4 binding comprises SEQ. ID. NO. 9.

28. The inducible expression system of claim 27 wherein the promoter region further comprises a sequence selected from a group consisting of SEQ. ID. NOS. 18 and 19.

29. The inducible expression system of claim 1 wherein the gene is codon optimized to express efficiently in an animal receiving the inducible expression system.

30. The inducible expression system of claim 1 wherein the second expression cassette is characterized by an undetectable basal expression of the gene, wherein the undetectable basal expression of the gene is characterized by an undetectable biological effect in the presence of the second expression cassette and in the absence of the ligand.

31. The inducible expression system of claim 1 wherein the system is characterized by an ability to be repetitively induced over a period of time with multiple introduction of the ligand.

32. The inducible expression system of claim 1 wherein the first and second expression cassette are part of a single nucleic acid vector.

33. The inducible expression system of claim 1 wherein the first expression cassette is part of a first plasmid vector and the second expression cassette is part of a second plasmid vector.

34. An inducible expression system comprising:

a first expression cassette having a tissue-specific promoter driving the expression of a nucleic acid sequence, wherein the nucleic acid sequence comprises:

a coding sequence encoding for

a mutated GAL-4 DNA-binding domain, wherein the mutation is found within amino acids 54-93 of SEQ. ID. NO. 10;

a transcription activator domain, and

a mutated ligand-binding domain of a progesterone receptor; wherein the mutation is found within amino acids 813 - 933 of human progesterone receptor;

a second expression cassette encoding a gene coding for a protein having GHRH activity; and

a ligand capable of binding to the mutated ligand-binding domain and activating expression of the gene.

35. The inducible expression system of claim 34 wherein the coding sequence is a DNA sequence that encodes for an amino acid sequence selected from the group consisting of a human GHRH, a porcine GHRH, a bovine GHRH, and functional truncated and protease resistant versions thereof.

36. A GHRH inducible expression system comprising:

a first plasmid having the sequence SEQ. ID. NO. 28, and

a second plasmid having the sequence SEQ. ID. NO. 29.

37. A composition comprising:

a pharmacological dose of a first plasmid having a first expression cassette and a second plasmid having a second expression cassette, wherein the first expression cassette comprises a nucleic acid sequence encoding for a fusion protein having the following domains:

a mutated GAL-4 DNA-binding domain;

a transregulatory domain; and

a mutated ligand-binding domain of a steroid-hormone receptor;

and wherein

the second expression cassette comprises a GHRH gene driven by an promoter inducible by the fusion protein.

38. A method of inducing the expression of a GHRH transgene, the method comprising the steps of:

(a) introducing an inducible expression system of claim 1 into a cell;

(b) inducing the expression of the gene by administering a ligand.

39. The method of claim 38 further comprising the step of electroporating the cell.

40. A method for regulated GHRH expression in vivo using the expression system of claims 1 or 34 for use in indications selected from a group consisting of: increasing weight; increasing lean body mass; decreasing fat mass; conversion to anabolism for a catabolic state associated with wasting; and increasing bone area, content and density.

41. The method of claim 40 wherein the wasting is associated with cancer, AIDS, burns, or post-surgery.

42. A method of regulated exposure to GHRH in vivo comprising the steps of:

(1) administering in vivo a regulated GHRH gene expression system;

(2) administering a pharmacologic dose of mifepristone on a pulsatile schedule, wherein the mifepristone regulates expression of the GHRH and the GHRH is expressed in vivo in a pulsatile fashion.

43. The method of claim 42 wherein the pulsatile schedule is a diurnal schedule.

44. Use of a regulated GHRH gene expression system according to claims 1 or 34 for the preparation of a pharmaceutical composition for indications selected from a group consisting of: increasing weight; increasing lean body mass; decreasing fat mass; conversion to anabolism for a catabolic state associated with wasting; and increasing bone area, content and density.

Figure 1

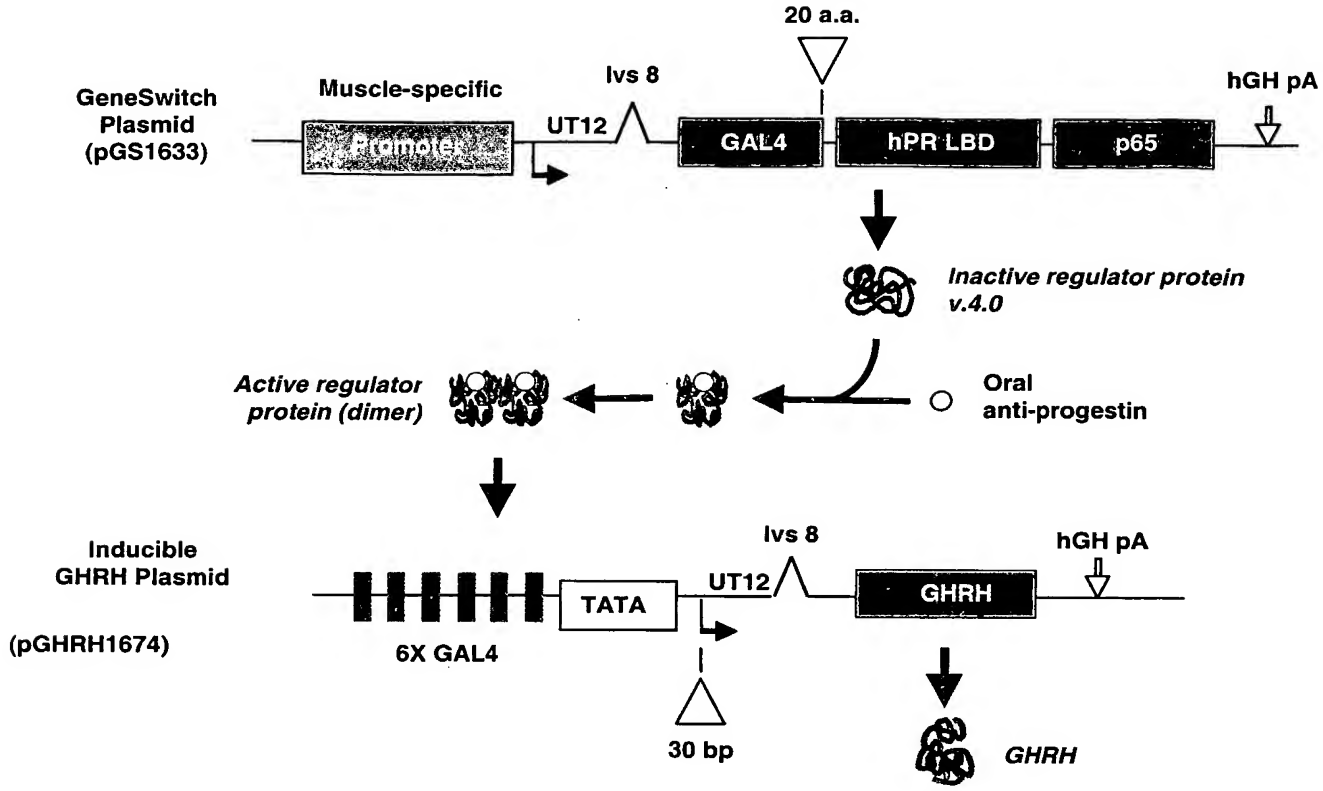


Figure 2

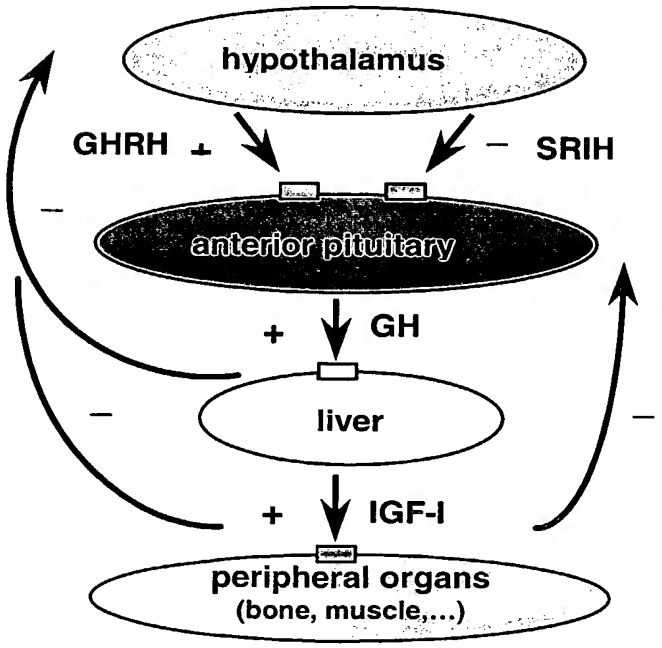


Figure 3

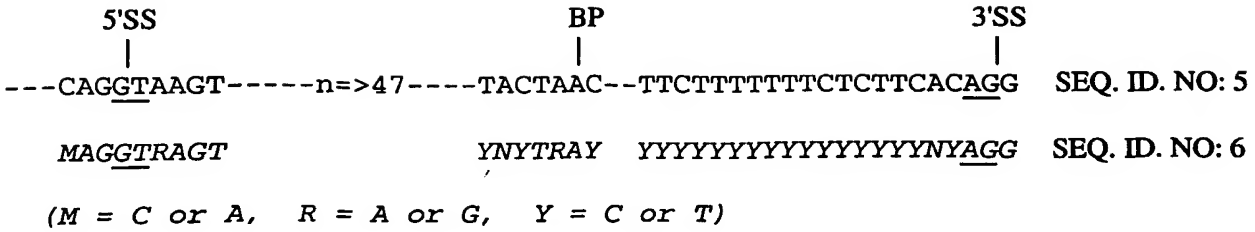


Figure 4

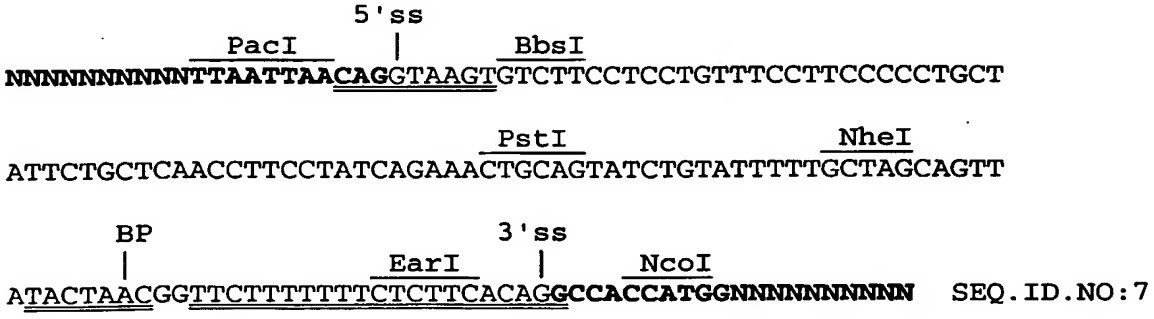
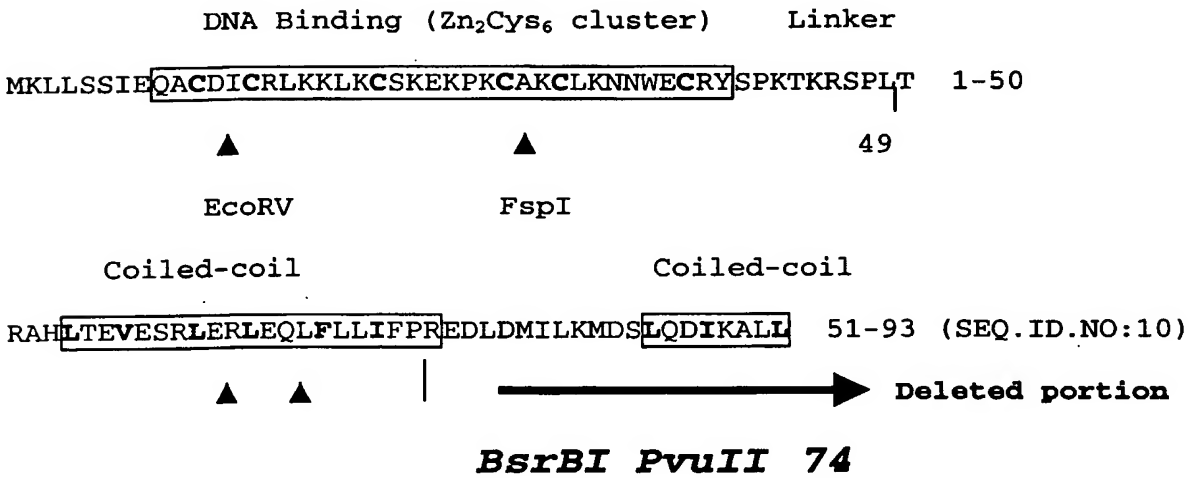


Figure 5



BsrBI PvuII 74

Figure 6A

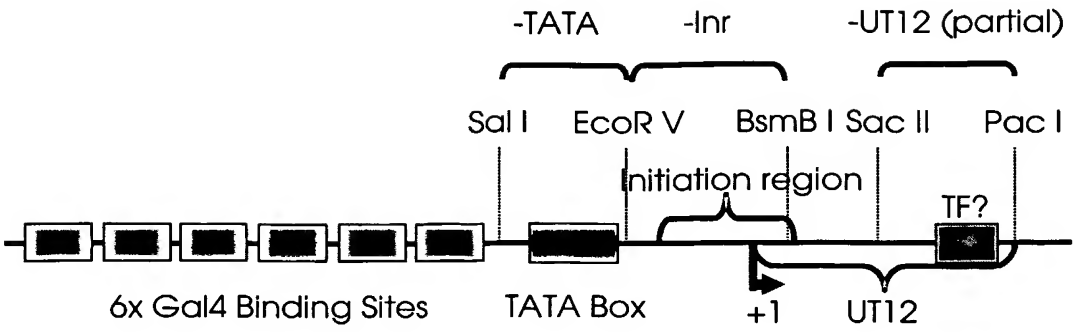


Figure 6B

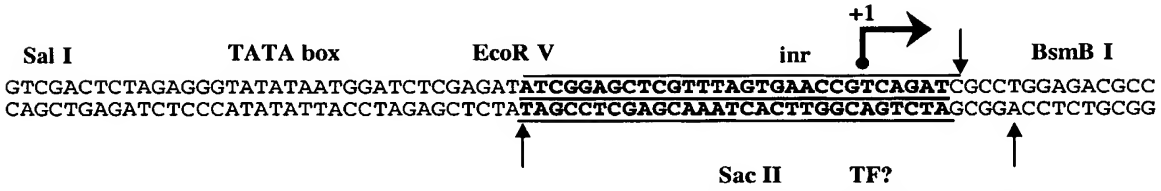
Inducible 6X GAL4/TATA Promoter (SEQ.ID.NO: 18)

..AAGCGGAGTACTGTCCTCCGAGTGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCGAGTCG

AGGGTCGAAGCGGAGTACTGTCCTCCGAGTGGAGTACTGTCCTCCGAGCGGAGTACTGTCCTCCG

AGTCGACTCTAGAGGGTATATAATGGATCTCGAGATATCGGAGCT ↓ CGTTTAGTGAACCGTC..

Figure 6C



Pac I

GATTCCCCGTGTTAATTAA
CTAAGGGGCACAATTAATT

(SEQ.ID.NO. 19)

Figure 7

GeneSwitch coding sequence

pGLVp65 – early version of regulator protein coding sequence (SEQ.ID.NO:12)
pGS1633 - GeneSwitch coding sequence version 4.0 (GAL4 domain underlined) (SEQ.ID. NO:13)

```
            1                                                                   70
{1210cds}   ATGGACTCCC AGCAGCCAGA TCTGAAGCTA CTGTCTTCTA TCGAACAAGC ATGCGATATT TGCCGACTTA
{1539cds}   ATGGACTCCC AGCAGCCAGA TCTGAAGCTA CTGTCTTCTA TCGAACAAGC ATGCGATATT TGCCGACTTA

            71                                                                  140
{1210cds}   AAAAGCTCAA GTGCTCCAAA GAAAAACCGA AGTGCGCCAA GTGTCTGAAG AACAACTGGG AGTGTCGCTA
{1539cds}   AAAAGCTCAA GTGCTCCAAA GAAAAACCGA AGTGCGCCAA GTGTCTGAAG AACAACTGGG AGTGTCGCTA

            141                                                                 210
{1210cds}   CTCTCCCAAA ACCAAAAGGT CTCCGCTGAC TAGGGCACAT CTGACAGAAG TGGAATCAAG GCTAGAAAGA
{1539cds}   CTCTCCCAAA ACCAAAAGGT CTCCGCTGAC TAGGGCACAT CTGACAGAAG TGGAATCAAG GCTAGAAAGA

            211                                                                 280
{1210cds}   CTGGAACAGC TATTTCTACT GATTTTTCCT CGAGAAGACC TTGACATGAT TTTGAAAATG GATTCTTTAC
{1539cds}   CTGGAACAGC TATTTCTACT GATTTTTCCT CGA ..................................

            281                                                                 350
{1210cds}   AGGATATAAA AGCATTGTTA GAATTCCCGG GTGTCGACCA GAAAAAGTTC AATAAAGTCA GAGTTGTGAG
{1539cds}   ....................................GACCA GAAAAAGTTC AATAAAGTCA GAGTTGTGAG

            351                                                                 420
{1210cds}   AGCACTGGAT GCTGTTGCTC TCCCACAGCC AGTGGGCGTT CCAAATGAAA GCCAAGCCCT AAGCCAGAGA
{1539cds}   AGCACTGGAT GCTGTTGCTC TCCCACAGCC AGTGGGCGTT CCAAATGAAA GCCAAGCCCT AAGCCAGAGA

            421                                                                 490
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{1539cds}   TTCACTTTTT CACCAGGTCA AGACATACAG TTGATTCCAC CACTGATCAA CCTGTTAATG AGCATTGAAC

            491                                                                 560
{1210cds}   CAGATGTGAT CTATGCAGGA CATGACAACA CAAAACCTGA CACCTCCAGT TCTTTGCTGA CAAGTCTTAA
{1539cds}   CAGATGTGAT CTATGCAGGA CATGACAACA CAAAACCTGA CACCTCCAGT TCTTTGCTGA CAAGTCTTAA

            561                                                                 630
{1210cds}   TCAACTAGGC GAGAGGCAAC TTCTTTCAGT AGTCAAGTGG TCTAAATCAT TGCCAGGTTT TCGAAACTTA
{1539cds}   TCAACTAGGC GAGAGGCAAC TTCTTTCAGT AGTCAAGTGG TCTAAATCAT TGCCAGGTTT TCGAAACTTA

            631                                                                 700
{1210cds}   CATATTGATG ACCAGATAAC TCTCATTCAG TATTCTTGGA TGAGCTTAAT GGTGTTTGGT CTAGGATGGA
{1539cds}   CATATTGATG ACCAGATAAC TCTCATTCAG TATTCTTGGA TGAGCTTAAT GGTGTTTGGT CTAGGATGGA

            701                                                                 770
{1210cds}   GATCCTACAA ACACGTCAGT GGGCAGATGC TGTATTTTGC ACCTGATCTA ATACTAAATG AACAGCGGAT
{1539cds}   GATCCTACAA ACACGTCAGT GGGCAGATGC TGTATTTTGC ACCTGATCTA ATACTAAATG AACAGCGGAT

            771                                                                 840
{1210cds}   GAAAGAATCA TCATTCTATT CATTATGCCT TACCATGTGG CAGATCCCAC AGGAGTTTGT CAAGCTTCAA
{1539cds}   GAAAGAATCA TCATTCTATT CATTATGCCT TACCATGTGG CAGATCCCAC AGGAGTTTGT CAAGCTTCAA

            841                                                                 910
{1210cds}   GTTAGCCAAG AAGAGTTCCT CTGTATGAAA GTATTGTTAC TTCTTAATAC AATTCCTTTG GAAGGGCTAC
{1539cds}   GTTAGCCAAG AAGAGTTCCT CTGTATGAAA GTATTGTTAC TTCTTAATAC AATTCCTTTG GAAGGGCTAC

            911                                                                 980
{1210cds}   GAAGTCAAAC CCAGTTTGAG GAGATGAGGT CAAGCTACAT TAGAGAGCTC ATCAAGGCAA TTGGTTTGAG
{1539cds}   GAAGTCAAAC CCAGTTTGAG GAGATGAGGT CAAGCTACAT TAGAGAGCTC ATCAAGGCAA TTGGTTTGAG

            981                                                                1050
{1210cds}   GCAAAAAGGA GTTGTGTCGA GCTCACAGCG TTTCTATCAA CTTACAAAAC TTCTTGATAA CTTGCATGAT
{1539cds}   GCAAAAAGGA GTTGTGTCGA GCTCACAGCG TTTCTATCAA CTTACAAAAC TTCTTGATAA CTTGCATGAT

            1051                                                               1120
{1210cds}   CTTGTCAAAC AACTTCATCT GTACTGCTTG AATACATTTA TCCAGTCCCG GGCACTGAGT GTTGAATTTC
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{1539cds}       CAGAAATGAT GTCTGAAGTT ATTGCTGGGT CGACGCCCAT GGAATTCCAG TACCTGCCAG ATACAGACGA

                1191                                                                                1260
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{1539cds}       TCGTCACCGG ATTGAGGAGA AACGTAAAAG GACATATGAG ACCTTCAAGA GCATCATGAA GAAGAGTCCT

                1261                                                                                1330
{1210cds}       TTCAGCGGAC CCACCGACCC CCGGCCTCCA CCTCGACGCA TTGCTGTGCC TTCCCGCAGC TCAGCTTCTG
{1539cds}       TTCAGCGGAC CCACCGACCC CCGGCCTCCA CCTCGACGCA TTGCTGTGCC TTCCCGCAGC TCAGCTTCTG

                1331                                                                                1400
{1210cds}       TCCCCAAGCC AGCACCCCAG CCCTATCCCT TTACGTCATC CCTGAGCACC ATCAACTATG ATGAGTTTCC
{1539cds}       TCCCCAAGCC AGCACCCCAG CCCTATCCCT TTACGTCATC CCTGAGCACC ATCAACTATG ATGAGTTTCC

                1401                                                                                1470
{1210cds}       CACCATGGTG TTTCCTTCTG GGCAGATCAG CCAGGCCTCG GCCTTGGCCC CGGCCCCTCC CCAAGTCCTG
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                1471                                                                                1540
{1210cds}       CCCCAGGCTC CAGCCCCTGC CCCTGCTCCA GCCATGGTAT CAGCTCTGGC CCAGGCCCCA GCCCCTGTCC
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                1541                                                                                1610
{1210cds}       CAGTCCTAGC CCCAGGCCCT CCTCAGGCTG TGGCCCCACC TGCCCCCAAG CCCACCCAGG CTGGGGAAGG
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                1611                                                                                1680
{1210cds}       AACGCTGTCA GAGGCCCTGC TGCAGCTGCA GTTTGATGAT GAAGACCTGG GGGCCTTGCT TGGCAACAGC
{1539cds}       AACGCTGTCA GAGGCCCTGC TGCAGCTGCA GTTTGATGAT GAAGACCTGG GGGCCTTGCT TGGCAACAGC

                1681                                                                                1750
{1210cds}       ACAGACCCAG CTGTGTTCAC AGACCTGGCA TCCGTCGACA ACTCCGAGTT TCAGCAGCTG CTGAACCAGG
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                1751                                                                                1820
{1210cds}       GCATACCTGT GGCCCCCCAC ACAACTGAGC CCATGCTGAT GGAGTACCCT GAGGCTATAA CTCGCCTAGT
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                1821                                                                                1890
{1210cds}       GACAGGGGCC CAGAGGCCCC CCGACCCAGC TCCTGCTCCA CTGGGGGCCC CGGGGCTCCC CAATGGCCTC
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                1891                                                                                1960
{1210cds}       CTTTCAGGAG ATGAAGACTT CTCCTCCATT GCGGACATGG ACTTCTCAGC CCTGCTGAGT CAGATCAGCT
{1539cds}       CTTTCAGGAG ATGAAGACTT CTCCTCCATT GCGGACATGG ACTTCTCAGC CCTGCTGAGT CAGATCAGCT

                1961
{1210cds}       CCTAA
{1539cds}       CCTAA
```

Figure 8

GeneSwitch amino acid sequence

pGLVp65 – early version of regulator protein coding sequence (SEQ.ID. NO: 14)
pGS1633 - GeneSwitch amino acid sequence v 4.0 (GAL4 domain underlined) (SEQ.ID. NO: 15)

```
        1                                                                   70
{1210}  MDSQQPDLKL LSSIEQACDI CRLKKLKCSK EKPKCAKCLK NNWECRYSPK TKRSPLTRAH LTEVESRLER
{1539}  MDSQQPDLKL LSSIEQACDI CRLKKLKCSK EKPKCAKCLK NNWECRYSPK TKRSPLTRAH LTEVESRLER

        71                                                                  140
{1210}  LEQLFLLIFP REDLDMILKM DSLQDIKALL EFPGVDQKKF NKVRVVRALD AVALPQPVGV PNESQALSQR
{1539}  LEQLFLLIFP R                      DQKKF NKVRVVRALD AVALPQPVGV PNESQALSQR

        141                                                                 210
{1210}  FTFSPGQDIQ LIPPLINLLM SIEPDVIYAG HDNTKPDTSS SLLTSLNQLG ERQLLSVVKW SKSLPGFRNL
{1539}  FTFSPGQDIQ LIPPLINLLM SIEPDVIYAG HDNTKPDTSS SLLTSLNQLG ERQLLSVVKW SKSLPGFRNL

        211                                                                 280
{1210}  HIDDQITLIQ YSWMSLMVFG LGWRSYKHVS GQMLYFAPDL ILNEQRMKES SFYSLCLTMW QIPQEFVKLQ
{1539}  HIDDQITLIQ YSWMSLMVFG LGWRSYKHVS GQMLYFAPDL ILNEQRMKES SFYSLCLTMW QIPQEFVKLQ

        281                                                                 350
{1210}  VSQEEFLCMK VLLLLNTIPL EGLRSQTQFE EMRSSYIREL IKAIGLRQKG VVSSSQRFYQ LTKLLDNLHD
{1539}  VSQEEFLCMK VLLLLNTIPL EGLRSQTQFE EMRSSYIREL IKAIGLRQKG VVSSSQRFYQ LTKLLDNLHD

        351                                                                 420
{1210}  LVKQLHLYCL NTFIQSRALS VEFPEMMSEV IAGSTPMEFQ YLPDTDDRHR IEEKRKRTYE TFKSIMKKSP
{1539}  LVKQLHLYCL NTFIQSRALS VEFPEMMSEV IAGSTPMEFQ YLPDTDDRHR IEEKRKRTYE TFKSIMKKSP

        421                                                                 490
{1210}  FSGPTDPRPP PRRIAVPSRS SASVPKPAPQ PYPFTSSLST INYDEFPTMV FPSGQISQAS ALAPAPPQVL
{1539}  FSGPTDPRPP PRRIAVPSRS SASVPKPAPQ PYPFTSSLST INYDEFPTMV FPSGQISQAS ALAPAPPQVL

        491                                                                 560
{1210}  PQAPAPAPAP AMVSALAQAP APVPVLAPGP PQAVAPPAPK PTQAGEGTLS EALLQLQFDD EDLGALLGNS
{1539}  PQAPAPAPAP AMVSALAQAP APVPVLAPGP PQAVAPPAPK PTQAGEGTLS EALLQLQFDD EDLGALLGNS

        561                                                                 630
{1210}  TDPAVFTDLA SVDNSEFQQL LNQGIPVAPH TTEPMLMEYP EAITRLVTGA QRPPDPAPAP LGAPGLPNGL
{1539}  TDPAVFTDLA SVDNSEFQQL LNQGIPVAPH TTEPMLMEYP EAITRLVTGA QRPPDPAPAP LGAPGLPNGL

        631          655
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{1539}  LSGDEDFSSI ADMDFSALLS QISS*
```

Figure 9

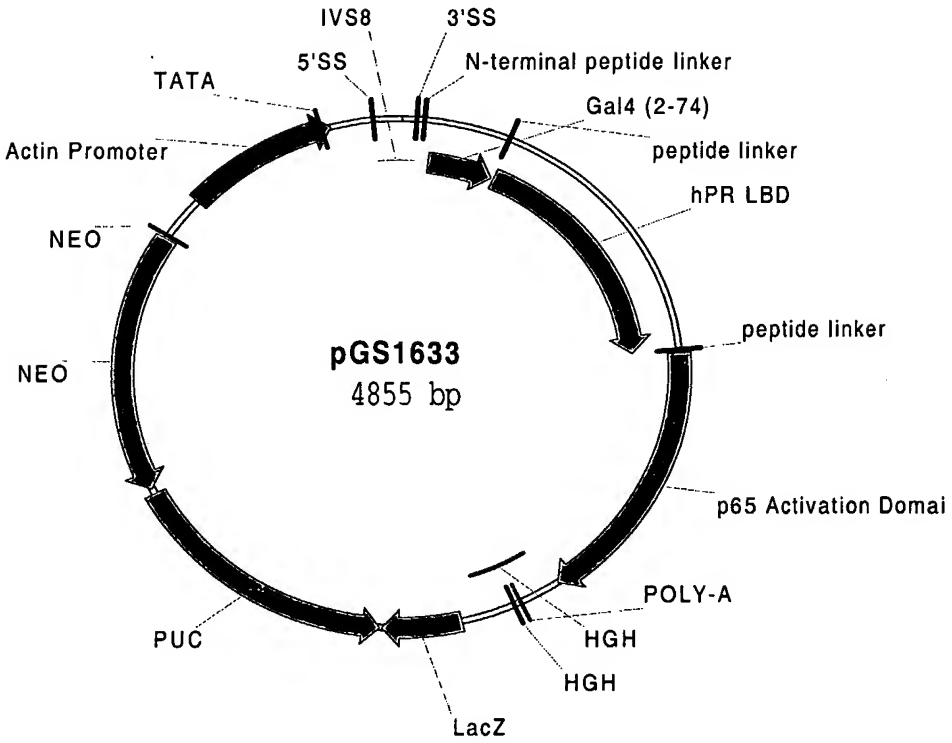

Figure 10

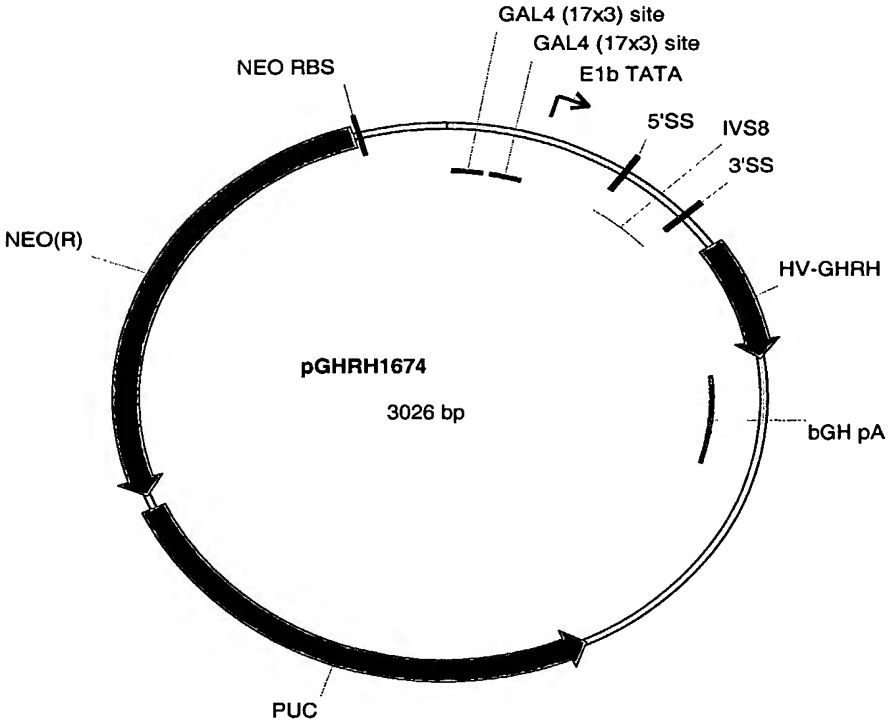

Figure 11

pGS1633, SEQ.ID.NO. 28

GAL4 DNA Binding Domain

Ligand Binding Domain

P65 transact. domain

```
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  61 catggactcc cagcagccag atctgaagct actgtcttct atcgaacaag catgcgatat
 121 ttgccgactt aaaaaagctc agtgctccaa agaaaaaccg caagtcgcca agtgtcgtaa
 181 gaacaactgg gagtgtcgct acctctcccaa aaccaaaagg tcctccgctga ctagggcaca
 241 tctgacagaa gtggaatcaa gggctagaaag actggaacag ctatttctag tgatttttcc
 301 tcgagaccag aaaaagttca ataaagtcag agttgtgaga gcactggatg ctgttgctct
 361 cccacagcca gtgggcgttc caaatgaaag ccaagcccta agccagagat tcactttttc
 421 accaggtcaa gacatacagt tgattccacc actgatcaac ctgttaatga gcattgaacc
 481 agatgtgatc tatgcaggac atgacaacac aaaacctgac acctccagtt ctttgctgac
 541 aagtcttaat caactaggcg agaggcaact tctttcagta gtcaagtggt ctaaatcatt
 601 gccaggtttt cgaaacttac atattgatga ccagataact ctcattcagt attcttggat
 661 gagcttaatg gtgtttggtc taggatggag atcctacaaa cacgtcagtg ggcagatgct
 721 gtattttgca cctgatctaa tactaaatga acagcggatg aaagaatcat cattctattc
 781 attatgcctt accatgtggc agatcccaca ggagtttgtc aagcttcaag ttagccaaga
 841 agagttcctc tgtatgaaag tattgttact tcttaataca attcctttgg aagggctacg
 901 aagtcaaacc cagtttgagg agatgaggtc aagctacatt agagagctca tcaaggcaat
 961 tggtttgagg caaaaaggag ttgtgtcgag ctcacagcgt ttctatcaac ttacaaaact
1021 tcttgataac ttgcatgatc ttgtcaaaca acttcatctg tactgcttga atacatttat
1081 ccagtcccgg gcactgagtg ttgaatttcc agaaatgatg tctgaagtta ttgctgggtc
1141 gacgcccatg gaattccagt tacctgccaga tacagacgat tcgtcaccgga ttgaggagaa
1201 acgtaaaagg tacatatgaga ccttcaagag catcatgaag taagagtcctt tcagcggacc
1261 caccgacccc cggcctccac ctcgacgcat tgctgtgcct tcccgcagct cagcttctgt
1321 ccccaagcca gcacccagcc cctatccctt tacgtcatcc ctgagcacca tcaactatga
1381 tgagtttccc accatggtgt ttccttctgg gcagatcagc caggcctcgg cctttggccca
1441 ggcccctccc caagtcctgc ccaggctccc agccccctgcc cctgctccag ccatggtatc
1501 agctctggcc cagaccccag ccctgtccc agtcctagcc ccaggccctc ctcaggctgt
1561 ggccccacct gccccccaagc ccaacccagg ctgggaagga acgctgtcag aggccctgct
1621 gcagctgcag tttgatgatg aagacctggg ggccttgctt ggcaacagca cagacccagc
1681 tgtgttcaca gacctggcat ccgtcgacaa ctccgagttt cagcagctgc tgaacctaggg
1741 catacctgtg gccccccaca caactgagcc catgctgatg gagtacccta aggctataac
1801 tcgcctagtg acagggggccc agaggccccc cgacccagct ccctgctcca cctgggggccc
1861 ggggctcccc aatggcctcc tttcaggaga tgaagacttc tcctccattg cggacatgga
1921 cttctcagcc ctgctgagtc agatcagctc ctaaggatcc tccggactag aaaagccgaa
1981 ttctgcagga attgggtggc atccctgtga cccctcccca gtgcctctcc tggccctgga
2041 agttgccact ccagtgccca ccagccttgt cctaataaaa ttaagttgca tcattttgtc
2101 tgactaggtg tccttctata atattatggg gtggaggggg gtggtatgga gcaaggggca
2161 agttgggaag acaacctgta gggctcgagg gggggcccgg taccagcttt tgttcccttt
2221 agtgagggtt aatttcgagc ttggcgtaat catggtcata gctgtttcct gtgtgaaatt
2281 gttatccgct cacaattcca cacaacatac gagccggaag cataaagtgt aaagcctggg
2341 gtgcctaatg agtgagctaa ctcacattaa ttgcgttgcg ctcactgccc gctttccagt
2401 cgggaaacct gtcgtgccag ctgcattaat gaatcggcca acgcgcgggg agaggcggtt
2461 tgcgtattgg gcgctcttcc gcttcctcgc tcactgactc gctgcgctcg gtcgttcggc
2521 tgcggcgagc ggtatcagct cactcaaagg cggtaatacg gttatccaca gaatcagggg
2581 ataacgcagg aaagaacatg tgagcaaaag gccagcaaaa ggccaggaac cgtaaaaagg
2641 ccgcgttgct ggcgtttttc cataggctcc gccccctga cgagcatcac aaaaatcgac
2701 gctcaagtca gaggtggcga aacccgacag gactataaag ataccaggcg tttccccctg
2761 gaagctccct cgtgcgctct cctgttccga ccctgccgct taccggatac ctgtccgcct
2821 ttctcccttc gggaagcgtg gcgctttctc atagctcacg ctgtaggtat ctcagttcgg
2881 tgtaggtcgt tcgctccaag ctgggctgtg tgcacgaacc ccccgttcag cccgaccgct
2941 gcgccttatc cggtaactat cgtcttgagt ccaacccggt aagacacgac ttatcgccac
3001 tggcagcagc cactggtaac aggattagca gagcgaggta tgtaggcggt gctacagagt
3061 tcttgaagtg gtggcctaac tacggctaca ctagaaggac agtatttggt atctgcgctc
3121 tgctgaagcc agttaccttc ggaaaaagag ttggtagctc ttgatccggc aaacaaacca
3181 ccgctggtag cggtggtttt tttgtttgca agcagcagat tacgcgcaga aaaaaaggat
3241 ctcaagaaga tcctttgatc ttttctacgg ggtctgacgc tcagaagaac tcgtcaagaa
3301 ggcgatagaa ggcgatgcgc tgcgaatcgg gagcggcgat accgtaaagc acgaggaagc
3361 ggtcagccca ttcgccgcca agctcttcag caatatcacg ggtagccaac gctatgtcct
3421 gatagcggtc cgccacaccc agccggccac agtcgatgaa tccagaaaag cggccatttt
3481 ccaccatgat attcggcaag caggcatcgc catgcgtcac gacgagatcc tcgccgtcgg
3541 gcatgcgcgc cttgagcctg gcgaacagtt cggctggcgc gagcccctga tgctcttcgt
3601 ccagatcatc ctgatcgaca agaccggctt ccatccgagt acgtgctcgc tcgatgcgat
3661 gtttcgcttg gtggtcgaat gggcggtag ccggatcaag cgtatgcagc cgccgcattg
3721 catcagccat gatggatact ttctcggcag gagcaaggtg agatgacagg agatcctgcc
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3901 cattcagggc accggacagg tcggtcttga caaaaagaac cgggcgcccc tgcgctgaca
3961 gccggaacac ggcggcatca gagcagccga ttgtctgttg tgcccagtca tagccgaata
4021 gcctctccac ccaagcggcc ggagaacctg cgtgcaatcc atcttgttca atcatgcgaa
4081 acgatcctca tcctgtctct tgatcagatc ttgatccccc gcgccatcag atccttggcg
4141 gcaagaaagc catccagttt actttgcagg gcttcccaac cttaccagag ggcgaattcg
4201 agcttgcatg cctgcagggg ccgctctagc tagagtctgc ctgccccctg cctggcacag
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4321 gccccgctgc cccccgaggc ctcctccctc acgcctcgct gcgctcccgg ctcccgcacg
4381 gccctgggag aggcccccac cgcttcgtcc ttaacgggcc cggcggtgcc gggggattat
4441 ttcggccccg gccccggggg ggcccggcag acgctcctta tacggcccgg cctcgctcac
4501 ctgggccgcg gccaggagcg ccttctttgg gcagcgccgg gccggggccg cgccgggccc
4561 gacacccaaa tatggcgacg gccggggccg cattcctggg ggccgggcgg tgctcccgcc
4621 cgcctcgata aaaggctccg gggccggcgg gcgactcaga tcgcctggag acgccatcca
4681 cgctgttttg acctccatag aagacaccgg gaccgatcca gcctccgcgg ccgggaacgg
4741 tgcattggaa cgcggattcc ccgtgttaat taacaggtaa gtgtcttcct cctgtttcct
4801 tcccctgcta ttctgctcaa ccttcctatc agaaactgca gtatctgtat ttttg
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Figure 12

pGHRH1674, SEQ.ID. 29

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   1  AGGGTCGAAG CGGAGTACTG TCCTCCGAGT GGAGTACTGT CCTCCGAGCG
      TCCCAGCTTC GCCTCATGAC AGGAGGCTCA CCTCATGACA GGAGGCTCGC

  51  GAGTACTGTC CTCCGAGTCG AGGGTCGAAG CGGAGTACTG TCCTCCGAGT
      CTCATGACAG GAGGCTCAGC TCCCAGCTTC GCCTCATGAC AGGAGGCTCA

 101  GGAGTACTGT CCTCCGAGCG GAGTACTGTC CTCCGAGTCG ACTCTAGAGG
      CCTCATGACA GGAGGCTCGC CTCATGACAG GAGGCTCAGC TGAGATCTCC

 151  GTATATAATG GATCTCGAGA TGCCTGGAGA CGCCATCCAC GCTGTTTTGA
      CATATATTAC CTAGAGCTCT ACGGACCTCT GCGGTAGGTG CGACAAAACT

 201  CCTCCATAGA AGACACCGGG ACCGATCCAG CCTCCGCGGC CGGGAACGGT
      GGAGGTATCT TCTGTGGCCC TGGCTAGGTC GGAGGCGCCG GCCCTTGCCA

 251  GCATTGGAAC GCGGATTCCC CGTGTTAATT AACAGGTAAG TGTCTTCCTC
      CGTAACCTTG CGCCTAAGGG GCACAATTAA TTGTCCATTC ACAGAAGGAG

 301  CTGTTTCCTT CCCCTGCTAT TCTGCTCAAC CTTCCTATCA GAAACTGCAG
      GACAAAGGAA GGGGACGATA AGACGAGTTG GAAGGATAGT CTTTGACGTC

 351  TATCTGTATT TTTGCTAGCA GTAATACTAA CGGTTCTTTT TTTCTCTTCA
      ATAGACATAA AAACGATCGT CATTATGATT GCCAAGAAAA AAAGAGAAGT

 401  CAGGCCACGA TCCCAAGGCC CAACTCCCCG AACCACTCAG GGTCCTGTGG
      GTCCGGTGCT AGGGTTCCGG GTTGAGGGGC TTGGTGAGTC CCAGGACACC

                                M   V   L   W   V   F   F   F   V   I   L  .
 451  ACAGCTCACC TAGCTGCCAT GGTGCTCTGG GTGTTCTTCT TTGTGATCCT
      TGTCGAGTGG ATCGACGGTA CCACGAGACC CACAAGAAGA AACACTAGGA

      .  T   L   S   N   S   S   H   C   S   P   P   P   P   L   T   L   R  .
 501  CACCCTCAGC AACAGCTCCC ACTGCTCCCC ACCTCCCCCT TTGACCCTCA
      GTGGGAGTCG TTGTCGAGGG TGACGAGGGG TGGAGGGGGA AACTGGGAGT

      .  M   R   R   H   V   D   A   I   F   T   N   S   Y   R   K   V
 551  GGATGCGGCG GCACGTAGAT GCCATCTTCA CCAACAGCTA CCGGAAGGTG
      CCTACGCCGC CGTGCATCTA CGGTAGAAGT GGTTGTCGAT GGCCTTCCAC

       L   A   Q   L   S   A   R   K   L   L   Q   D   I   L   N   R   Q
 601  CTGGCCCAGC TGTCCGCCCG CAAGCTGCTC CAGGACATCC TGAACAGGCA
      GACCGGGTCG ACAGGCGGGC GTTCGACGAG GTCCTGTAGG ACTTGTCCGT

      .  Q   G   E   R   N   Q   E   Q   G   A   T
 651  GCAGGGAGAG AGGAACCAAG AGCAAGGAGC ATAATGACTG CAGGAATTCG
      CGTCCCTCTC TCCTTGGTTC TCGTTCCTCG TATTACTGAC GTCCTTAAGC

 701  ATATCAAGCT CGCTGATCAG CCTCGACTGT GCCTTCTAGT TGCCAGCCAT
      TATAGTTCGA GCGACTAGTC GGAGCTGACA CGGAAGATCA ACGGTCGGTA

 751  CTGTTGTTTG CCCCTCCCCC GTGCCTTCCT TGACCCTGGA AGGTGCCACT
      GACAACAAAC GGGGAGGGGG CACGGAAGGA ACTGGGACCT TCCACGGTGA

 801  CCCACTGTCC TTTCCTAATA AAATGAGGAA ATTGCATCGC ATTGTCTGAG
      GGGTGACAGG AAAGGATTAT TTTACTCCTT TAACGTAGCG TAACAGACTC

 851  TAGGTGTCAT TCTATTCTGG GGGGTGGGGT GGGGCAGGAC AGCAAGGGGG
      ATCCACAGTA AGATAAGACC CCCCACCCCA CCCCGTCCTG TCGTTCCCCC

 901  AGGATTGGGA AGACAATAGC AGGCATGCTG GGGATGCGGT GGGCTCTATG
      TCCTAACCCT TCTGTTATCG TCCGTACGAC CCCTACGCCA CCCGAGATAC

 951  GCTTCTGAGG CGGAAAGAAC CAGCTGGGGC TCGAGCATGC AAGCTTCGAG
      CGAAGACTCC GCCTTTCTTG GTCGACCCCG AGCTCGTACG TTCGAAGCTC
```

Protease resistant porcine GHRH pre-pro coding sequence including signal peptide

The mature GHRH sequence is shaded

```
1001    GGGGGGCCCG GTACCAGCTT TTGTTCCCTT TAGTGAGGGT TAATTTCGAG
        CCCCCCGGGC CATGGTCGAA AACAAGGGAA ATCACTCCCA ATTAAAGCTC

1051    CTTGGCGTAA TCATGGTCAT AGCTGTTTCC TGTGTGAAAT TGTTATCCGC
        GAACCGCATT AGTACCAGTA TCGACAAAGG ACACACTTTA ACAATAGGCG

1101    TCACAATTCC ACACAACATA CGAGCCGGAA GCATAAAGTG TAAAGCCTGG
        AGTGTTAAGG TGTGTTGTAT GCTCGGCCTT CGTATTTCAC ATTTCGGACC

1151    GGTGCCTAAT GAGTGAGCTA ACTCACATTA ATTGCGTTGC GCTCACTGCC
        CCACGGATTA CTCACTCGAT TGAGTGTAAT TAACGCAACG CGAGTGACGG

1201    CGCTTTCCAG TCGGGAAACC TGTCGTGCCA GCTGCATTAA TGAATCGGCC
        GCGAAAGGTC AGCCCTTTGG ACAGCACGGT CGACGTAATT ACTTAGCCGG

1251    AACGCGCGGG GAGAGGCGGT TTGCGTATTG GGCGCTCTTC CGCTTCCTCG
        TTGCGCGCCC CTCTCCGCCA AACGCATAAC CCGCGAGAAG GCGAAGGAGC

1301    CTCACTGACT CGCTGCGCTC GGTCGTTCGG CTGCGGCGAG CGGTATCAGC
        GAGTGACTGA GCGACGCGAG CCAGCAAGCC GACGCCGCTC GCCATAGTCG

1351    TCACTCAAAG GCGGTAATAC GGTTATCCAC AGAATCAGGG GATAACGCAG
        AGTGAGTTTC CGCCATTATG CCAATAGGTG TCTTAGTCCC CTATTGCGTC

1401    GAAAGAACAT GTGAGCAAAA GGCCAGCAAA AGGCCAGGAA CCGTAAAAAG
        CTTTCTTGTA CACTCGTTTT CCGGTCGTTT TCCGGTCCTT GGCATTTTTC

1451    GCCGCGTTGC TGGCGTTTTT CCATAGGCTC CGCCCCCCTG ACGAGCATCA
        CGGCGCAACG ACCGCAAAAA GGTATCCGAG GCGGGGGGAC TGCTCGTAGT

1501    CAAAAATCGA CGCTCAAGTC AGAGGTGGCG AAACCCGACA GGACTATAAA
        GTTTTTAGCT GCGAGTTCAG TCTCCACCGC TTTGGGCTGT CCTGATATTT

1551    GATACCAGGC GTTTCCCCCT GGAAGCTCCC TCGTGCGCTC TCCTGTTCCG
        CTATGGTCCG CAAAGGGGGA CCTTCGAGGG AGCACGCGAG AGGACAAGGC

1601    ACCCTGCCGC TTACCGGATA CCTGTCCGCC TTTCTCCCTT CGGGAAGCGT
        TGGGACGGCG AATGGCCTAT GGACAGGCGG AAAGAGGGAA GCCCTTCGCA

1651    GGCGCTTTCT CATAGCTCAC GCTGTAGGTA TCTCAGTTCG GTGTAGGTCG
        CCGCGAAAGA GTATCGAGTG CGACATCCAT AGAGTCAAGC CACATCCAGC

1701    TTCGCTCCAA GCTGGGCTGT GTGCACGAAC CCCCCGTTCA GCCCGACCGC
        AAGCGAGGTT CGACCCGACA CACGTGCTTG GGGGGCAAGT CGGGCTGGCG

1751    TGCGCCTTAT CCGGTAACTA TCGTCTTGAG TCCAACCCGG TAAGACACGA
        ACGCGGAATA GGCCATTGAT AGCAGAACTC AGGTTGGGCC ATTCTGTGCT

1801    CTTATCGCCA CTGGCAGCAG CCACTGGTAA CAGGATTAGC AGAGCGAGGT
        GAATAGCGGT GACCGTCGTC GGTGACCATT GTCCTAATCG TCTCGCTCCA

1851    ATGTAGGCGG TGCTACAGAG TTCTTGAAGT GGTGGCCTAA CTACGGCTAC
        TACATCCGCC ACGATGTCTC AAGAACTTCA CCACCGGATT GATGCCGATG

1901    ACTAGAAGGA CAGTATTTGG TATCTGCGCT CTGCTGAAGC CAGTTACCTT
        TGATCTTCCT GTCATAAACC ATAGACGCGA GACGACTTCG GTCAATGGAA

1951    CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACC ACCGCTGGTA
        GCCTTTTTCT CAACCATCGA GAACTAGGCC GTTTGTTTGG TGGCGACCAT

2001    GCGGTGGTTT TTTTGTTTGC AAGCAGCAGA TTACGCGCAG AAAAAAAGGA
        CGCCACCAAA AAAACAAACG TTCGTCGTCT AATGCGCGTC TTTTTTTCCT

2051    TCTCAAGAAG ATCCTTTGAT CTTTTCTACG GGGTCTGACG CTCAGAAGAA
        AGAGTTCTTC TAGGAAACTA GAAAAGATGC CCCAGACTGC GAGTCTTCTT

2101    CTCGTCAAGA AGGCGATAGA AGGCGATGCG CTGCGAATCG GGAGCGGCGA
        GAGCAGTTCT TCCGCTATCT TCCGCTACGC GACGCTTAGC CCTCGCCGCT

2151    TACCGTAAAG CACGAGGAAG CGGTCAGCCC ATTCGCCGCC AAGCTCTTCA
        ATGGCATTTC GTGCTCCTTC GCCAGTCGGG TAAGCGGCGG TTCGAGAAGT
```

```
2201    GCAATATCAC GGGTAGCCAA CGCTATGTCC TGATAGCGGT CCGCCACACC
        CGTTATAGTG CCCATCGGTT GCGATACAGG ACTATCGCCA GGCGGTGTGG

2251    CAGCCGGCCA CAGTCGATGA ATCCAGAAAA GCGGCCATTT TCCACCATGA
        GTCGGCCGGT GTCAGCTACT TAGGTCTTTT CGCCGGTAAA AGGTGGTACT

2301    TATTCGGCAA GCAGGCATCG CCATGCGTCA CGACGAGATC CTCGCCGTCG
        ATAAGCCGTT CGTCCGTAGC GGTACGCAGT GCTGCTCTAG GAGCGGCAGC

2351    GGCATGCGCG CCTTGAGCCT GGCGAACAGT TCGGCTGGCG CGAGCCCCTG
        CCGTACGCGC GGAACTCGGA CCGCTTGTCA AGCCGACCGC GCTCGGGGAC

2401    ATGCTCTTCG TCCAGATCAT CCTGATCGAC AAGACCGGCT TCCATCCGAG
        TACGAGAAGC AGGTCTAGTA GGACTAGCTG TTCTGGCCGA AGGTAGGCTC

2451    TACGTGCTCG CTCGATGCGA TGTTTCGCTT GGTGGTCGAA TGGGCAGGTA
        ATGCACGAGC GAGCTACGCT ACAAAGCGAA CCACCAGCTT ACCCGTCCAT

2501    GCCGGATCAA GCGTATGCAG CCGCCGCATT GCATCAGCCA TGATGGATAC
        CGGCCTAGTT CGCATACGTC GGCGGCGTAA CGTAGTCGGT ACTACCTATG

2551    TTTCTCGGCA GGAGCAAGGT GAGATGACAG GAGATCCTGC CCCGGCACTT
        AAAGAGCCGT CCTCGTTCCA CTCTACTGTC CTCTAGGACG GGGCCGTGAA

2601    CGCCCAATAG CAGCCAGTCC CTTCCCGCTT CAGTGACAAC GTCGAGCACA
        GCGGGTTATC GTCGGTCAGG GAAGGGCGAA GTCACTGTTG CAGCTCGTGT

2651    GCTGCGCAAG GAACGCCCGT CGTGGCCAGC CACGATAGCC GCGCTGCCTC
        CGACGCGTTC CTTGCGGGCA GCACCGGTCG GTGCTATCGG CGCGACGGAG

2701    GTCCTGCAGT TCATTCAGGG CACCGGACAG GTCGGTCTTG ACAAAAAGAA
        CAGGACGTCA AGTAAGTCCC GTGGCCTGTC CAGCCAGAAC TGTTTTTCTT

2751    CCGGGCGCCC CTGCGCTGAC AGCCGGAACA CGGCGGCATC AGAGCAGCCG
        GGCCCGCGGG GACGCGACTG TCGGCCTTGT GCCGCCGTAG TCTCGTCGGC

2801    ATTGTCTGTT GTGCCCAGTC ATAGCCGAAT AGCCTCTCCA CCCAAGCGGC
        TAACAGACAA CACGGGTCAG TATCGGCTTA TCGGAGAGGT GGGTTCGCCG

2851    CGGAGAACCT GCGTGCAATC CATCTTGTTC AATCATGCGA AACGATCCTC
        GCCTCTTGGA CGCACGTTAG GTAGAACAAG TTAGTACGCT TTGCTAGGAG

2901    ATCCTGTCTC TTGATCAGAT CTTGATCCCC TGCGCCATCA GATCCTTGGC
        TAGGACAGAG AACTAGTCTA GAACTAGGGG ACGCGGTAGT CTAGGAACCG

2951    GGCAAGAAAG CCATCCAGTT TACTTTGCAG GGCTTCCCAA CCTTACCAGA
        CCGTTCTTTC GGTAGGTCAA ATGAAACGTC CCGAAGGGTT GGAATGGTCT

3001    GGGCGAATTC GAGCTTGCAT GCCTGC
        CCCGCTTAAG CTCGAACGTA CGGACG
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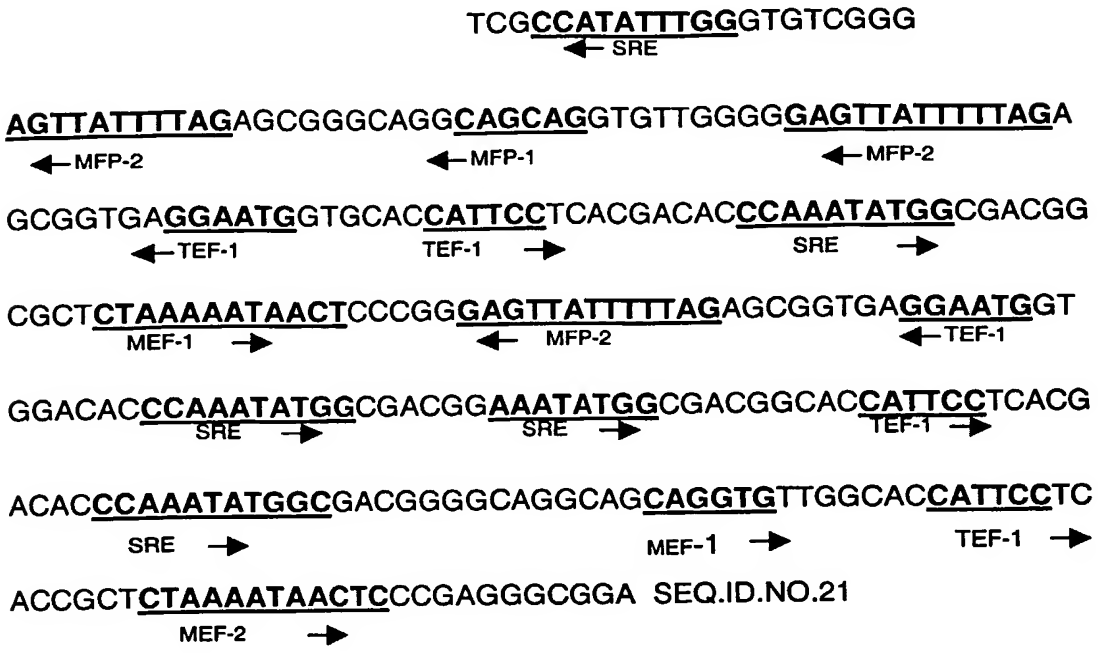
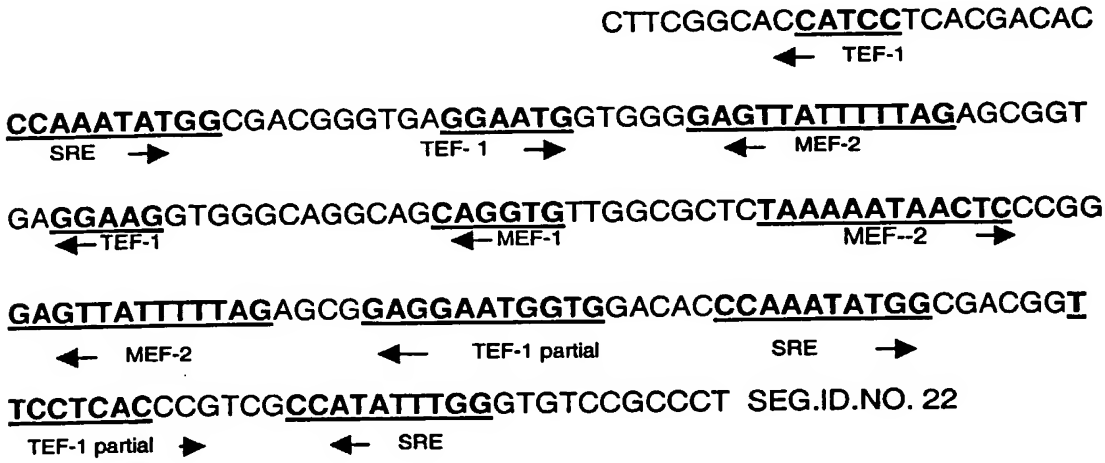
Figure 13

C1-28 Synthetic Muscle Specific Promoter



C5-12 Synthetic Muscle Specific Promoter



SRE	5'----GACACCCAAATATGGCGACGG----3'	21 mer SEQ.ID.NO. 23
MEF-1	5'----CCAACACCTGCTGCCTGCC----3'	19 mer SEQ.ID.NO. 24
MEF-2	5'----CGCTCTAAAAATAACTCCC----3'	19 mer SEQ.ID.NO. 25
TEF-1	5'----CACCATTCCTCAC----3'	13 mer SEQ.ID.NO. 26
SP1	5'----CCGTCCGCCCTCGG----3'	14 mer SEQ.ID.NO. 27

Figure 14

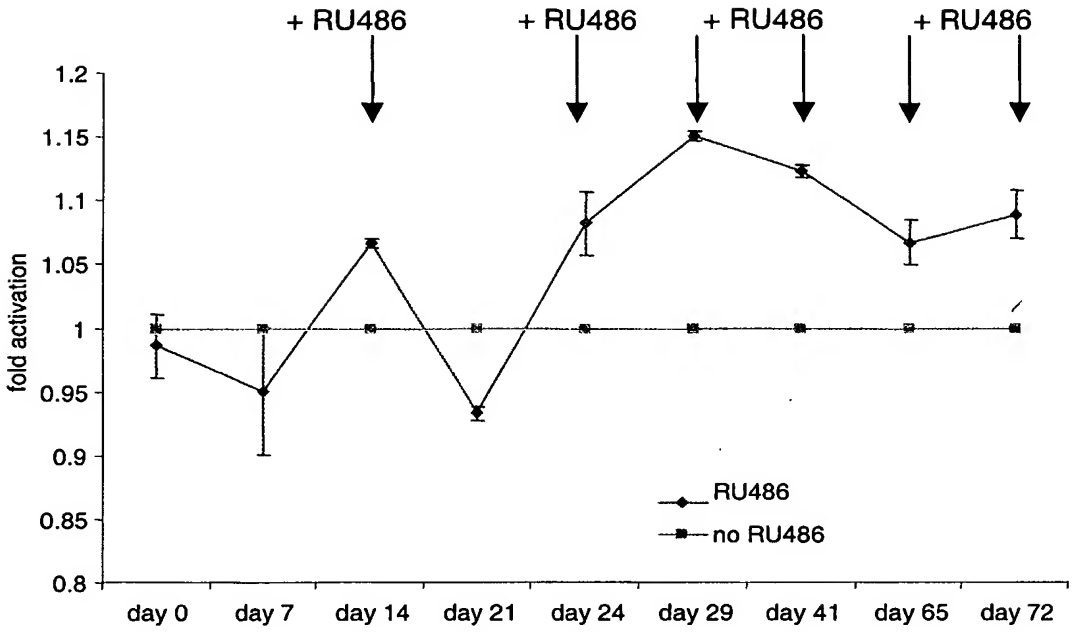


Figure 15

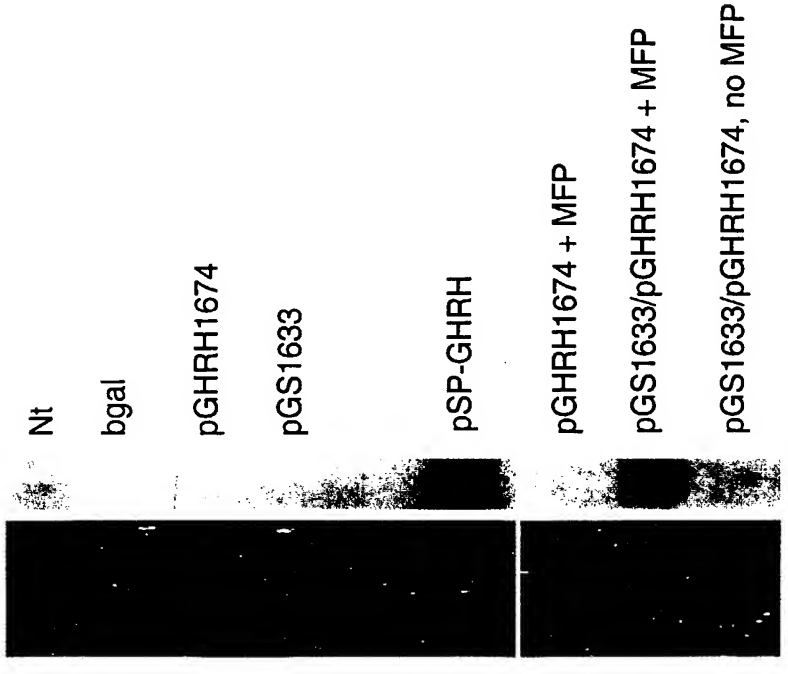


Figure 16

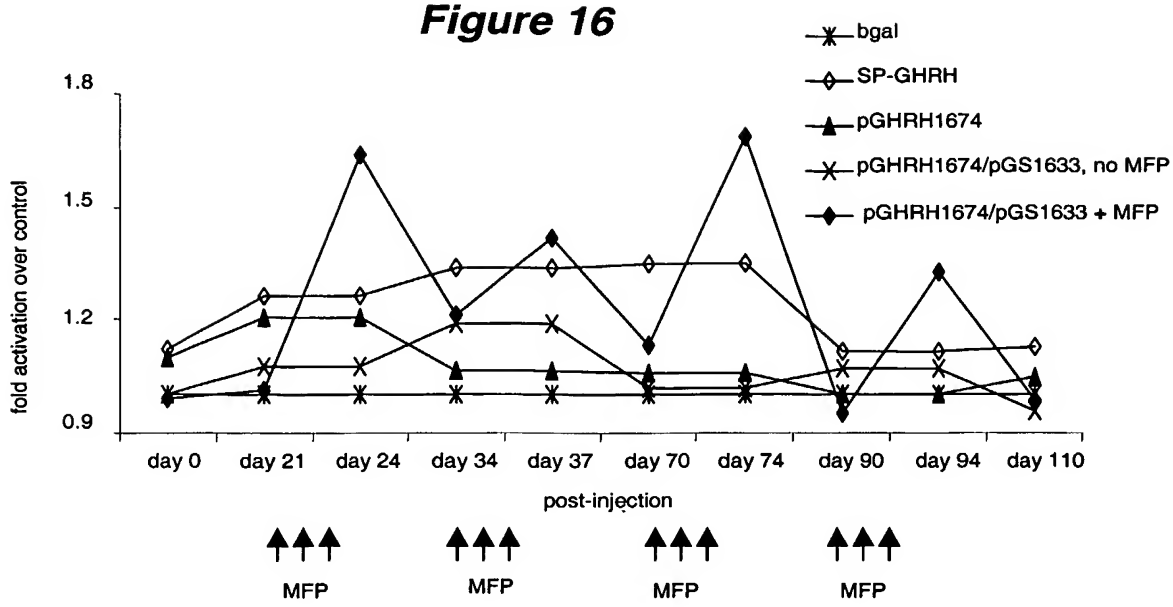


Figure 17

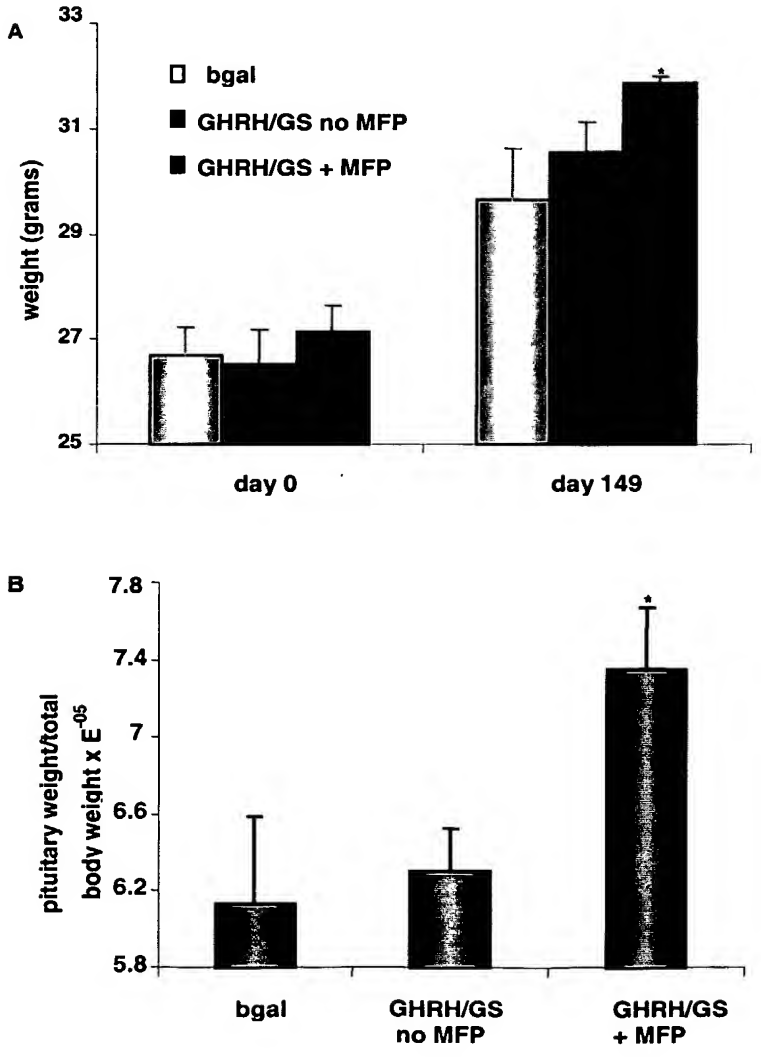


Figure 18

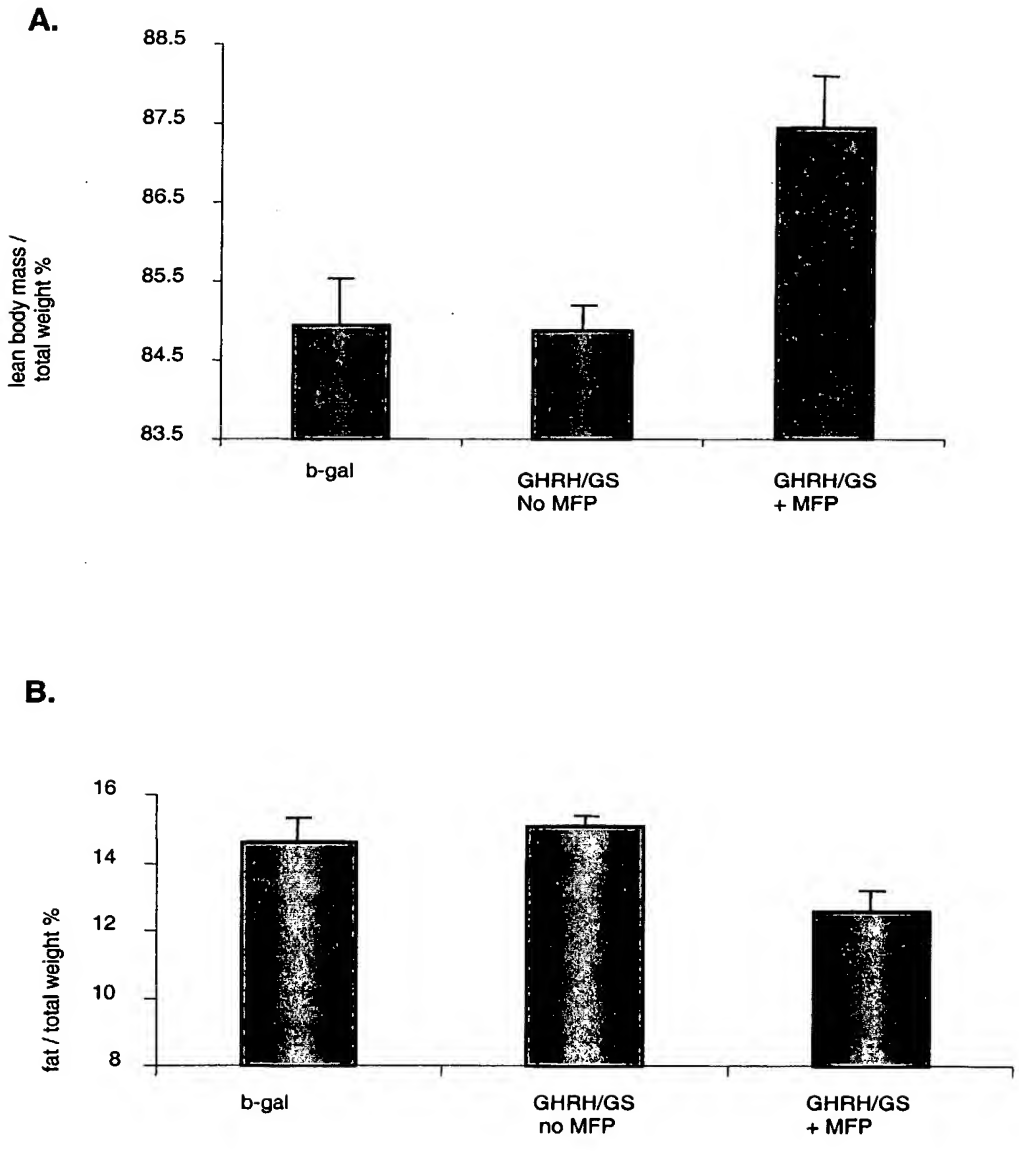
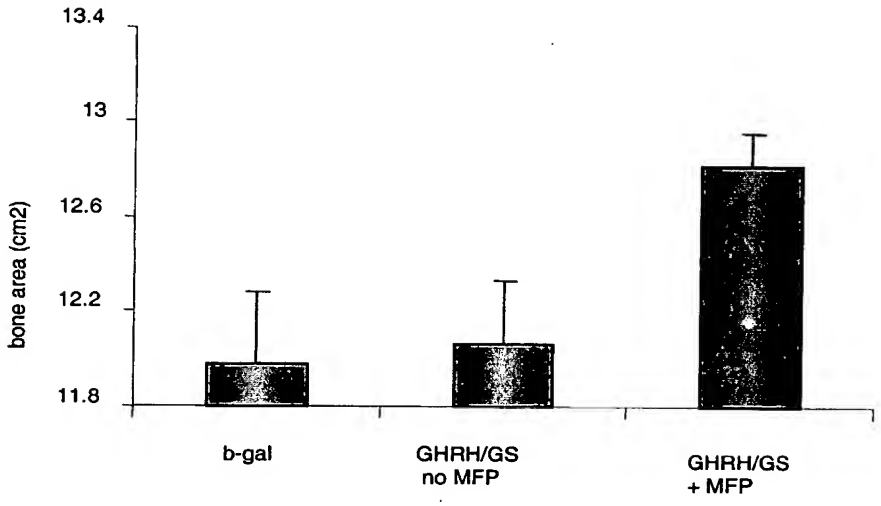

A.
88.5
87.5
86.5
85.5
84.5
83.5
lean body mass / total weight %
b-gal
GHRH/GS No MFP
GHRH/GS + MFP
B.
16
14
12
10
8
fat / total weight %
b-gal
GHRH/GS no MFP
GHRH/GS + MFP

Figure 19

A.


13.4
13
12.6
12.2
11.8
bone area (cm2)
b-gal
GHRH/GS
no MFP
GHRH/GS
+ MFP

B.

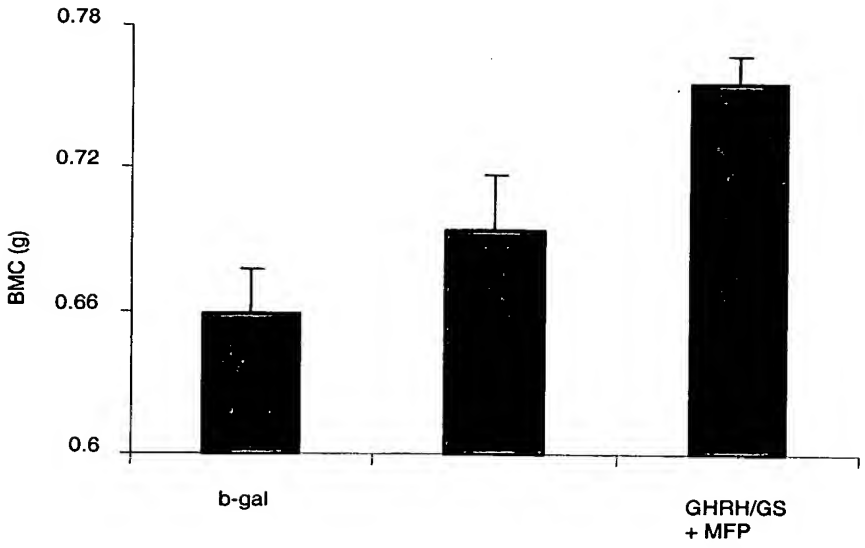

0.78
0.72
0.66
0.6
BMC (g)
b-gal
GHRH/GS
+ MFP

Figure 20

A.

ggatcccaaggcccaactccccgaaccactcagggtcctgtggacagctcacctagctgcc**atg**gtgctctgggtgttct
tctttgtgatcctcaccctcagcaacagctcccactgctccccacctccccctttgaccctcaggatgcggcgg**tat**gca
gatgccatcttcaccaacagctaccggaaggtgctgggccagctgtccgcccgcaagctgctccaggacatcatgagcag
gcagcagggagagagcaaccaagagcgaggagca**taatga**ctgcaggaattcgatatcaagctt (SEQ.ID.NO.30)

Human 1-40 hydroxy sequence optimized codon BamHI/ HindIII fragment

The pre-pro hormone starts at atg (bold) and the mature GHRH starts at Tyr 1 (tat codon, bold and shadowed). The sequence stops at taatga (bold).

B.

YADAIFTNSY RKVLGQLSAR	KLLQDIMSRQ	QGESNQERGA	RARL	44 aa human GHRH (SEQ.ID.NO 31)
YADAIFTNSY RKVLGQLSAR	KLLQDIMSRQ	QGESNQERGA	OH	human -OH 40 aa (SEQ.ID.NO. 32)
---------- ----------	----------	---R---Q--	OH	porcine wt (SEQ.ID.NO. 33)
HV-------- ----A-----	------LN--	---R---Q--	OH	HVsuper porcine (SEQ.ID.NO. 34)

SEQUENCE LISTING

<110> Nordstrom, Jeffrey
Draghia-Akli, Ruxandra

<120> REGULATED EXPRESSION OF GHRH

<130> 265/042 PCT

<140> Not Yet Assigned
<141> 2001-05-30

<160> 34

<170> PatentIn version 3.0

<210> 1
<211> 9
<212> RNA
<213> Artificial

<220>
<223> 5' splice site sequence of a synthetic intron.

<400> 1
cagguaagu 9

<210> 2
<211> 9
<212> RNA
<213> Homo sapiens

<400> 2
guccauuca 9

<210> 3
<211> 7
<212> RNA
<213> Artificial

<220>
<223> Branch point sequence for a synthetic intron.

<400> 3
uacuaac 7

<210> 4
<211> 6
<212> RNA
<213> Homo sapiens

<400> 4
augaug 6

<210> 5
<211> 86
<212> DNA

<213> Artificial

<220>
<223> A general synthetic intron sequence where intronic sequences are represented by N.

<220>
<221> Intron
<222> (1)..(86)
<223> N can be any base and can be longer or shorter than depicted

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aacnnttctt tttttctctt cacagg 86

<210> 6
<211> 86
<212> DNA
<213> Artificial

<220>
<223> Consensus intron sequence wherein intronic sequences are represented by N.

<220>
<221> Intron
<222> (1)..(86)
<223> N can be any base and can be longer or shorter than depicted

<400> 6
maggtragtn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnynyt 60

raynnyyyyy yyyyyyyyyy ynyagg 86

<210> 7
<211> 160
<212> DNA
<213> Artificial

<220>
<223> A synthetic intron termed IVS8 where exonic sequences are represented by N.

<220>
<221> Intron
<222> (1)..(160)
<223> N can be any base and can be longer or shorter than depicted

<400> 7
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gctcaacctt cctatcagaa actgcagtat ctgtattttt gctagcagtt atactaacgg 120

ttctttttt ctcttcacag gccaccatgg nnnnnnnnnn 160

<210> 8
<211> 110
<212> DNA
<213> Artificial

<220>
<223> 5' untranslated region derived from CMV.

<400> 8
tcagatcgcc tggagacgcc atccacgctg ttttgacctc catagaagac accgggaccg 60

atccagcctc cgcggccggg aacggtgcat tggaacgcgg attccccgtg 110

<210> 9
<211> 17
<212> DNA
<213> Saccharomyces cerevisiae

<400> 9
cggaagactc tcctccg 17

<210> 10
<211> 93
<212> PRT
<213> Saccharomyces cerevisiae

<400> 10

Met Lys Leu Leu Ser Ser Ile Glu Gln Ala Cys Asp Ile Cys Arg Leu
1 5 10 15

Lys Lys Leu Lys Cys Ser Lys Glu Lys Pro Lys Cys Ala Lys Cys Leu
20 25 30

Lys Asn Asn Trp Glu Cys Arg Tyr Ser Pro Lys Thr Lys Arg Ser Pro
35 40 45

Leu Thr Arg Ala His Leu Thr Glu Val Glu Ser Arg Leu Glu Arg Leu
50 55 60

Glu Gln Leu Phe Leu Leu Ile Phe Pro Arg Glu Asp Leu Asp Met Ile
65 70 75 80

Leu Lys Met Asp Ser Leu Gln Asp Ile Lys Ala Leu Leu
85 90

<210> 11
<211> 28
<212> PRT
<213> Artificial

<220>
<223> General sequence for GAL4 DNA recognition unit having cysteine-rich amino acid sequence forming two alpha helices that bind to two Zn ions.

<220>
<221> HELIX
<222> (1)..(28)
<223> X can be any amino acid residue.

<400> 11

Cys Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa
1 5 10 15

Xaa Cys Xaa Xaa Cys Xaa Xaa Xaa Xaa Xaa Xaa Cys
20 25

<210> 12
<211> 1965
<212> DNA
<213> Artificial

<220>
<223> Coding Sequence for the GeneSwitch regulator protein v.3.1 comprising a GAL-4 DBD, a NFkBp65 transactivation domain and a mutated hPR LBD.

<400> 12
atggactccc agcagccaga tctgaagcta ctgtcttcta tcgaacaagc atgcgatatt 60

tgccgactta aaaagctcaa gtgctccaaa gaaaaaccga agtgcgccaa gtgtctgaag 120

aacaactggg agtgtcgcta ctctcccaaa accaaaaggt ctccgctgac tagggcacat 180

ctgacagaag tggaatcaag gctagaaaga ctggaacagc tatttctact gatttttcct 240

cgagaagacc ttgacatgat tttgaaaatg gattctttac aggatataaa agcattgtta 300

gaattcccgg gtgtcgacca gaaaaagttc aataaagtca gagttgtgag agcactggat 360

gctgttgctc tcccacagcc agtgggcgtt ccaaatgaaa gccaagccct aagccagaga 420

ttcacttttt caccaggtca agacatacag ttgattccac cactgatcaa cctgttaatg 480

agcattgaac cagatgtgat ctatgcagga catgacaaca caaaacctga cacctccagt 540

tctttgctga caagtcttaa tcaactaggc gagaggcaac ttctttcagt agtcaagtgg 600

tctaaatcat tgccaggttt tcgaaactta catattgatg accagataac tctcattcag 660

tattcttgga tgagcttaat ggtgtttggt ctaggatgga gatcctacaa acacgtcagt 720

gggcagatgc tgtattttgc acctgatcta atactaaatg aacagcggat gaaagaatca 780

tcattctatt cattatgcct taccatgtgg cagatcccac aggagtttgt caagcttcaa 840

gttagccaag aagagttcct ctgtatgaaa gtattgttac ttcttaatac aattcctttg 900

gaagggctac gaagtcaaac ccagtttgag gagatgaggt caagctacat tagagagctc 960

atcaaggcaa ttggtttgag gcaaaaagga gttgtgtcga gctcacagcg tttctatcaa 1020

cttacaaaac ttcttgataa cttgcatgat cttgtcaaac aacttcatct gtactgcttg 1080

aatacattta tccagtcccg ggcactgagt gttgaatttc cagaaatgat gtctgaagtt 1140

attgctgggt cgacgcccat ggaattccag tacctgccag atacagacga tcgtcaccgg 1200

attgaggaga aacgtaaaag gacatatgag accttcaaga gcatcatgaa gaagagtcct 1260

ttcagcggac ccaccgaccc ccggcctcca cctcgacgca ttgctgtgcc ttcccgcagc 1320

tcagcttctg tccccaagcc agcaccccag ccctatccct ttacgtcatc cctgagcacc 1380

atcaactatg atgagtttcc caccatggtg tttccttctg ggcagatcag ccaggcctcg 1440

gccttggccc cggcccctcc ccaagtcctg ccccaggctc cagcccctgc ccctgctcca 1500

gccatggtat cagctctggc ccaggcccca gcccctgtcc cagtcctagc cccaggccct 1560

cctcaggctg tggccccacc tgcccccaag cccacccagg ctggggaagg aacgctgtca 1620

gaggccctgc tgcagctgca gtttgatgat gaagacctgg gggccttgct tggcaacagc 1680

acagacccag ctgtgttcac agacctggca tccgtcgaca actccgagtt tcagcagctg 1740

ctgaaccagg gcatacctgt ggcccccccac acaactgagc ccatgctgat ggagtaccct 1800

gaggctataa ctcgcctagt gacaggggcc cagaggcccc ccgacccagc tcctgctcca 1860

ctgggggccc cggggctccc caatggcctc ctttcaggag atgaagactt ctcctccatt 1920

gcggacatgg acttctcagc cctgctgagt cagatcagct cctaa 1965

<210> 13
<211> 1893
<212> DNA
<213> Artificial

<220>
<223> Coding Sequence for GeneSwitch Regulator protein v.4.0 comprising a truncated GAL4 DBD, p65 transactivation domain and mutated hPR LBD.

<400> 13
atggactccc agcagccaga tctgaagcta ctgtcttcta tcgaacaagc atgcgatatt 60

tgccgactta aaaagctcaa gtgctccaaa gaaaaaccga agtgcgccaa gtgtctgaag 120

aacaactggg agtgtcgcta ctctcccaaa accaaaaggt ctccgctgac tagggcacat 180

ctgacagaag tggaatcaag gctagaaaga ctggaacagc tatttctact gatttttcct 240

cgagaccaga aaaagttcaa taaagtcaga gttgtgagag cactggatgc tgttgctctc 300

ccacagccag tgggcgttcc aaatgaaagc caagccctaa gccagagatt cacttttca 360

ccaggtcaag acatacagtt gattccacca ctgatcaacc tgttaatgag cattgaacca 420

gatgtgatct atgcaggaca tgacaacaca aaacctgaca cctccagttc tttgctgaca 480

agtcttaatc aactaggcga gaggcaactt ctttcagtag tcaagtggtc taaatcattg 540

ccaggttttc gaaacttaca tattgatgac cagataactc tcattcagta ttcttggatg 600

agcttaatgg tgtttggtct aggatggaga tcctacaaac acgtcagtgg gcagatgctg 660

tattttgcac ctgatctaat actaaatgaa cagcggatga aagaatcatc attctattca 720

ttatgcctta ccatgtggca gatcccacag gagtttgtca agcttcaagt tagccaagaa 780

gagttcctct gtatgaaagt attgttactt cttaatacaa ttcctttgga agggctacga 840

agtcaaaccc agtttgagga gatgaggtca agctacatta gagagctcat caaggcaatt 900

ggtttgaggc aaaaaggagt tgtgtcgagc tcacagcgtt tctatcaact tacaaaactt 960

cttgataact tgcatgatct tgtcaaacaa cttcatctgt actgcttgaa tacatttatc 1020

cagtcccggg cactgagtgt tgaatttcca gaaatgatgt ctgaagttat tgctgggtcg 1080

acgcccatgg aattccagta cctgccagat acagacgatc gtcaccggat tgaggagaaa 1140

cgtaaaagga catatgagac cttcaagagc atcatgaaga agagtccttt cagcggaccc 1200

accgaccccc ggcctccacc tcgacgcatt gctgtgcctt cccgcagctc agcttctgtc 1260

cccaagccag caccccagcc ctatcccttt acgtcatccc tgagcaccat caactatgat 1320

gagtttccca ccatggtgtt tccttctggg cagatcagcc aggcctcggc cttggccccg 1380

gcccctcccc aagtcctgcc ccaggctcca gcccctgccc ctgctccagc catggtatca 1440

gctctggccc aggccccagc ccctgtccca gtcctagccc caggccctcc tcaggctgtg 1500

gccccacctg cccccaagcc cacccaggct ggggaaggaa cgctgtcaga ggccctgctg 1560

cagctgcagt ttgatgatga agacctgggg gccttgcttg gcaacagcac agacccagct 1620

gtgttcacag acctggcatc cgtcgacaac tccgagtttc agcagctgct gaaccagggc 1680

atacctgtgg ccccccacac aactgagccc atgctgatgg agtaccctga ggctataact 1740

cgcctagtga caggggccca gaggcccccc gacccagctc ctgctccact gggggccccg 1800

gggctcccca atggcctcct ttcaggagat gaagacttct cctccattgc ggacatggac 1860

ttctcagccc tgctgagtca gatcagctcc taa 1893

<210> 14
<211> 654
<212> PRT
<213> Artificial

<220>
<223> Amino acid sequence of GeneSwitch regulator protein v.3.1 comprising GAL4 DBD, p65 transactivation domain and mutated hPR LBD.

<400> 14

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Met Asp Ser Gln Gln Pro Asp Leu Lys Leu Leu Ser Ser Ile Glu Gln
1               5                  10                  15

Ala Cys Asp Ile Cys Arg Leu Lys Lys Leu Lys Cys Ser Lys Glu Lys
            20                  25                  30

Pro Lys Cys Ala Lys Cys Leu Lys Asn Asn Trp Glu Cys Arg Tyr Ser
        35                  40                  45

Pro Lys Thr Lys Arg Ser Pro Leu Thr Arg Ala His Leu Thr Glu Val
    50                  55                  60

Glu Ser Arg Leu Glu Arg Leu Glu Gln Leu Phe Leu Leu Ile Phe Pro
65                  70                  75                  80

Arg Glu Asp Leu Asp Met Ile Leu Lys Met Asp Ser Leu Gln Asp Ile
                85                  90                  95

Lys Ala Leu Leu Glu Phe Pro Gly Val Asp Gln Lys Lys Phe Asn Lys
            100                 105                 110

Val Arg Val Val Arg Ala Leu Asp Ala Val Ala Leu Pro Gln Pro Val
        115                 120                 125

Gly Val Pro Asn Glu Ser Gln Ala Leu Ser Gln Arg Phe Thr Phe Ser
    130                 135                 140

Pro Gly Gln Asp Ile Gln Leu Ile Pro Pro Leu Ile Asn Leu Leu Met
145                 150                 155                 160

Ser Ile Glu Pro Asp Val Ile Tyr Ala Gly His Asp Asn Thr Lys Pro
                165                 170                 175

Asp Thr Ser Ser Ser Leu Leu Thr Ser Leu Asn Gln Leu Gly Glu Arg
            180                 185                 190

Gln Leu Leu Ser Val Val Lys Trp Ser Lys Ser Leu Pro Gly Phe Arg
        195                 200                 205

Asn Leu His Ile Asp Asp Gln Ile Thr Leu Ile Gln Tyr Ser Trp Met
    210                 215                 220

Ser Leu Met Val Phe Gly Leu Gly Trp Arg Ser Tyr Lys His Val Ser
225                 230                 235                 240

Gly Gln Met Leu Tyr Phe Ala Pro Asp Leu Ile Leu Asn Glu Gln Arg
                245                 250                 255

Met Lys Glu Ser Ser Phe Tyr Ser Leu Cys Leu Thr Met Trp Gln Ile
            260                 265                 270

Pro Gln Glu Phe Val Lys Leu Gln Val Ser Gln Glu Glu Phe Leu Cys
        275                 280                 285

Met Lys Val Leu Leu Leu Leu Asn Thr Ile Pro Leu Glu Gly Leu Arg
    290                 295                 300

Ser Gln Thr Gln Phe Glu Glu Met Arg Ser Ser Tyr Ile Arg Glu Leu
305                 310                 315                 320
```

```
Ile Lys Ala Ile Gly Leu Arg Gln Lys Gly Val Val Ser Ser Ser Gln
                325                 330                 335

Arg Phe Tyr Gln Leu Thr Lys Leu Leu Asp Asn Leu His Asp Leu Val
            340                 345                 350

Lys Gln Leu His Leu Tyr Cys Leu Asn Thr Phe Ile Gln Ser Arg Ala
        355                 360                 365

Leu Ser Val Glu Phe Pro Glu Met Met Ser Glu Val Ile Ala Gly Ser
    370                 375                 380

Thr Pro Met Glu Phe Gln Tyr Leu Pro Asp Thr Asp Asp Arg His Arg
385                 390                 395                 400

Ile Glu Glu Lys Arg Lys Arg Thr Tyr Glu Thr Phe Lys Ser Ile Met
                405                 410                 415

Lys Lys Ser Pro Phe Ser Gly Pro Thr Asp Pro Arg Pro Pro Pro Arg
            420                 425                 430

Arg Ile Ala Val Pro Ser Arg Ser Ser Ala Ser Val Pro Lys Pro Ala
        435                 440                 445

Pro Gln Pro Tyr Pro Phe Thr Ser Ser Leu Ser Thr Ile Asn Tyr Asp
    450                 455                 460

Glu Phe Pro Thr Met Val Phe Pro Ser Gly Gln Ile Ser Gln Ala Ser
465                 470                 475                 480

Ala Leu Ala Pro Ala Pro Pro Gln Val Leu Pro Gln Ala Pro Ala Pro
                485                 490                 495

Ala Pro Ala Pro Ala Met Val Ser Ala Leu Ala Gln Ala Pro Ala Pro
            500                 505                 510

Val Pro Val Leu Ala Pro Gly Pro Pro Gln Ala Val Ala Pro Pro Ala
        515                 520                 525

Pro Lys Pro Thr Gln Ala Gly Glu Gly Thr Leu Ser Glu Ala Leu Leu
    530                 535                 540

Gln Leu Gln Phe Asp Asp Glu Asp Leu Gly Ala Leu Leu Gly Asn Ser
545                 550                 555                 560

Thr Asp Pro Ala Val Phe Thr Asp Leu Ala Ser Val Asp Asn Ser Glu
                565                 570                 575

Phe Gln Gln Leu Leu Asn Gln Gly Ile Pro Val Ala Pro His Thr Thr
            580                 585                 590

Glu Pro Met Leu Met Glu Tyr Pro Glu Ala Ile Thr Arg Leu Val Thr
        595                 600                 605

Gly Ala Gln Arg Pro Pro Asp Pro Ala Pro Ala Pro Leu Gly Ala Pro
    610                 615                 620

Gly Leu Pro Asn Gly Leu Leu Ser Gly Asp Glu Asp Phe Ser Ser Ile
625                 630                 635                 640
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Ala Asp Met Asp Phe Ser Ala Leu Leu Ser Gln Ile Ser Ser
645 650

<210> 15
<211> 630
<212> PRT
<213> Artificial

<220>
<223> Amino acid sequence for GeneSwitch regulator protein v.4.0 comprising truncated GAL4 DBD, p65 transactivation domain and mutated hPR LBD.

<400> 15

Met Asp Ser Gln Gln Pro Asp Leu Lys Leu Leu Ser Ser Ile Glu Gln
1 5 10 15

Ala Cys Asp Ile Cys Arg Leu Lys Lys Leu Lys Cys Ser Lys Glu Lys
20 25 30

Pro Lys Cys Ala Lys Cys Leu Lys Asn Asn Trp Glu Cys Arg Tyr Ser
35 40 45

Pro Lys Thr Lys Arg Ser Pro Leu Thr Arg Ala His Leu Thr Glu Val
50 55 60

Glu Ser Arg Leu Glu Arg Leu Glu Gln Leu Phe Leu Leu Ile Phe Pro
65 70 75 80

Arg Asp Gln Lys Lys Phe Asn Lys Val Arg Val Val Arg Ala Leu Asp
85 90 95

Ala Val Ala Leu Pro Gln Pro Val Gly Val Pro Asn Glu Ser Gln Ala
100 105 110

Leu Ser Gln Arg Phe Thr Phe Ser Pro Gly Gln Asp Ile Gln Leu Ile
115 120 125

Pro Pro Leu Ile Asn Leu Leu Met Ser Ile Glu Pro Asp Val Ile Tyr
130 135 140

Ala Gly His Asp Asn Thr Lys Pro Asp Thr Ser Ser Ser Leu Leu Thr
145 150 155 160

Ser Leu Asn Gln Leu Gly Glu Arg Gln Leu Leu Ser Val Val Lys Trp
165 170 175

Ser Lys Ser Leu Pro Gly Phe Arg Asn Leu His Ile Asp Asp Gln Ile
180 185 190

Thr Leu Ile Gln Tyr Ser Trp Met Ser Leu Met Val Phe Gly Leu Gly
195 200 205

Trp Arg Ser Tyr Lys His Val Ser Gly Gln Met Leu Tyr Phe Ala Pro
210 215 220

Asp Leu Ile Leu Asn Glu Gln Arg Met Lys Glu Ser Ser Phe Tyr Ser
225 230 235 240

```
Leu Cys Leu Thr Met Trp Gln Ile Pro Gln Glu Phe Val Lys Leu Gln
                245                 250                 255

Val Ser Gln Glu Glu Phe Leu Cys Met Lys Val Leu Leu Leu Leu Asn
            260                 265                 270

Thr Ile Pro Leu Glu Gly Leu Arg Ser Gln Thr Gln Phe Glu Glu Met
        275                 280                 285

Arg Ser Ser Tyr Ile Arg Glu Leu Ile Lys Ala Ile Gly Leu Arg Gln
    290                 295                 300

Lys Gly Val Val Ser Ser Ser Gln Arg Phe Tyr Gln Leu Thr Lys Leu
305                 310                 315                 320

Leu Asp Asn Leu His Asp Leu Val Lys Gln Leu His Leu Tyr Cys Leu
                325                 330                 335

Asn Thr Phe Ile Gln Ser Arg Ala Leu Ser Val Glu Phe Pro Glu Met
            340                 345                 350

Met Ser Glu Val Ile Ala Gly Ser Thr Pro Met Glu Phe Gln Tyr Leu
        355                 360                 365

Pro Asp Thr Asp Asp Arg His Arg Ile Glu Glu Lys Arg Lys Arg Thr
    370                 375                 380

Tyr Glu Thr Phe Lys Ser Ile Met Lys Lys Ser Pro Phe Ser Gly Pro
385                 390                 395                 400

Thr Asp Pro Arg Pro Pro Pro Arg Arg Ile Ala Val Pro Ser Arg Ser
                405                 410                 415

Ser Ala Ser Val Pro Lys Pro Ala Pro Gln Pro Tyr Pro Phe Thr Ser
            420                 425                 430

Ser Leu Ser Thr Ile Asn Tyr Asp Glu Phe Pro Thr Met Val Phe Pro
        435                 440                 445

Ser Gly Gln Ile Ser Gln Ala Ser Ala Leu Ala Pro Ala Pro Pro Gln
    450                 455                 460

Val Leu Pro Gln Ala Pro Ala Pro Ala Pro Ala Pro Ala Met Val Ser
465                 470                 475                 480

Ala Leu Ala Gln Ala Pro Ala Pro Val Pro Val Leu Ala Pro Gly Pro
                485                 490                 495

Pro Gln Ala Val Ala Pro Pro Ala Pro Lys Pro Thr Gln Ala Gly Glu
            500                 505                 510

Gly Thr Leu Ser Glu Ala Leu Leu Gln Leu Gln Phe Asp Asp Glu Asp
        515                 520                 525

Leu Gly Ala Leu Leu Gly Asn Ser Thr Asp Pro Ala Val Phe Thr Asp
    530                 535                 540

Leu Ala Ser Val Asp Asn Ser Glu Phe Gln Gln Leu Leu Asn Gln Gly
545                 550                 555                 560
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Ile Pro Val Ala Pro His Thr Thr Glu Pro Met Leu Met Glu Tyr Pro
                565                 570                 575

Glu Ala Ile Thr Arg Leu Val Thr Gly Ala Gln Arg Pro Pro Asp Pro
            580                 585                 590

Ala Pro Ala Pro Leu Gly Ala Pro Gly Leu Pro Asn Gly Leu Leu Ser
        595                 600                 605

Gly Asp Glu Asp Phe Ser Ser Ile Ala Asp Met Asp Phe Ser Ala Leu
    610                 615                 620

Leu Ser Gln Ile Ser Ser
625                 630
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<210> 16
<211> 648
<212> PRT
<213> Artificial

<220>
<223> General amino acid sequence for GeneSwitch regulator protein v.4.0 comprising truncated GAL4 DBD, 065 transactivation domain and mutated hPR LBD. Xaa can be shorter or longer than depicted

<220>
<221> MISC_FEATURE
<222> (2)..(11)
<223> Xaa can be any amino acid residue

<220>
<221> MISC_FEATURE
<222> (85)..(94)
<223> Xaa can be any amino acid residue

<220>
<221> MISC_FEATURE
<222> (370)..(379)
<223> Xaa can be any amino acid residue

<400> 16

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Met Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Lys Leu Leu Ser Ser
1               5                   10                  15

Ile Glu Gln Ala Cys Asp Ile Cys Arg Leu Lys Lys Leu Lys Cys Ser
            20                  25                  30

Lys Glu Lys Pro Lys Cys Ala Lys Cys Leu Lys Asn Asn Trp Glu Cys
        35                  40                  45

Arg Tyr Ser Pro Lys Thr Lys Arg Ser Pro Leu Thr Arg Ala His Leu
    50                  55                  60

Thr Glu Val Glu Ser Arg Leu Glu Arg Leu Glu Gln Leu Phe Leu Leu
65                  70                  75                  80
```

```
Ile Phe Pro Arg Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Lys Lys
                85                  90                  95

Phe Asn Lys Val Arg Val Val Arg Ala Leu Asp Ala Val Ala Leu Pro
            100                 105                 110

Gln Pro Val Gly Val Pro Asn Glu Ser Gln Ala Leu Ser Gln Arg Phe
        115                 120                 125

Thr Phe Ser Pro Gly Gln Asp Ile Gln Leu Ile Pro Pro Leu Ile Asn
    130                 135                 140

Leu Leu Met Ser Ile Glu Pro Asp Val Ile Tyr Ala Gly His Asp Asn
145                 150                 155                 160

Thr Lys Pro Asp Thr Ser Ser Ser Leu Leu Thr Ser Leu Asn Gln Leu
                165                 170                 175

Gly Glu Arg Gln Leu Leu Ser Val Val Lys Trp Ser Lys Ser Leu Pro
            180                 185                 190

Gly Phe Arg Asn Leu His Ile Asp Asp Gln Ile Thr Leu Ile Gln Tyr
        195                 200                 205

Ser Trp Met Ser Leu Met Val Phe Gly Leu Gly Trp Arg Ser Tyr Lys
    210                 215                 220

His Val Ser Gly Gln Met Leu Tyr Phe Ala Pro Asp Leu Ile Leu Asn
225                 230                 235                 240

Glu Gln Arg Met Lys Glu Ser Ser Phe Tyr Ser Leu Cys Leu Thr Met
                245                 250                 255

Trp Gln Ile Pro Gln Glu Phe Val Lys Leu Gln Val Ser Gln Glu Glu
            260                 265                 270

Phe Leu Cys Met Lys Val Leu Leu Leu Leu Asn Thr Ile Pro Leu Glu
        275                 280                 285

Gly Leu Arg Ser Gln Thr Gln Phe Glu Glu Met Arg Ser Ser Tyr Ile
    290                 295                 300

Arg Glu Leu Ile Lys Ala Ile Gly Leu Arg Gln Lys Gly Val Val Ser
305                 310                 315                 320

Ser Ser Gln Arg Phe Tyr Gln Leu Thr Lys Leu Leu Asp Asn Leu His
                325                 330                 335

Asp Leu Val Lys Gln Leu His Leu Tyr Cys Leu Asn Thr Phe Ile Gln
            340                 345                 350

Ser Arg Ala Leu Ser Val Glu Phe Pro Glu Met Met Ser Glu Val Ile
        355                 360                 365

Ala Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Pro Met Glu Phe Gln
    370                 375                 380

Tyr Leu Pro Asp Thr Asp Asp Arg His Arg Ile Glu Glu Lys Arg Lys
385                 390                 395                 400
```

```
Arg Thr Tyr Glu Thr Phe Lys Ser Ile Met Lys Lys Ser Pro Phe Ser
                405                 410                 415

Gly Pro Thr Asp Pro Arg Pro Pro Pro Arg Arg Ile Ala Val Pro Ser
            420                 425                 430

Arg Ser Ser Ala Ser Val Pro Lys Pro Ala Pro Gln Pro Tyr Pro Phe
        435                 440                 445

Thr Ser Ser Leu Ser Thr Ile Asn Tyr Asp Glu Phe Pro Thr Met Val
    450                 455                 460

Phe Pro Ser Gly Gln Ile Ser Gln Ala Ser Ala Leu Ala Pro Ala Pro
465                 470                 475                 480

Pro Gln Val Leu Pro Gln Ala Pro Ala Pro Ala Pro Ala Pro Ala Met
                485                 490                 495

Val Ser Ala Leu Ala Gln Ala Pro Ala Pro Val Pro Val Leu Ala Pro
            500                 505                 510

Gly Pro Pro Gln Ala Val Ala Pro Pro Ala Pro Lys Pro Thr Gln Ala
        515                 520                 525

Gly Glu Gly Thr Leu Ser Glu Ala Leu Leu Gln Leu Gln Phe Asp Asp
    530                 535                 540

Glu Asp Leu Gly Ala Leu Leu Gly Asn Ser Thr Asp Pro Ala Val Phe
545                 550                 555                 560

Thr Asp Leu Ala Ser Val Asp Asn Ser Glu Phe Gln Gln Leu Leu Asn
                565                 570                 575

Gln Gly Ile Pro Val Ala Pro His Thr Thr Glu Pro Met Leu Met Glu
            580                 585                 590

Tyr Pro Glu Ala Ile Thr Arg Leu Val Thr Gly Ala Gln Arg Pro Pro
        595                 600                 605

Asp Pro Ala Pro Ala Pro Leu Gly Ala Pro Gly Leu Pro Asn Gly Leu
    610                 615                 620

Leu Ser Gly Asp Glu Asp Phe Ser Ser Ile Ala Asp Met Asp Phe Ser
625                 630                 635                 640

Ala Leu Leu Ser Gln Ile Ser Ser
                645
```

<210> 17
<211> 630
<212> PRT
<213> Artificial

<220>
<223> Amino acid sequence for GeneSwitch regulator protein v.4.0 comprising truncated GAL4 DBD, p65 transactivation domain and mutated hPR LBD.

<400> 17

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Met Asp Ser Gln Gln Pro Asp Leu Lys Leu Leu Ser Ser Ile Glu Gln
1               5                  10                  15

Ala Cys Asp Ile Cys Arg Leu Lys Lys Leu Lys Cys Ser Lys Glu Lys
            20                  25                  30

Pro Lys Cys Ala Lys Cys Leu Lys Asn Asn Trp Glu Cys Arg Tyr Ser
        35                  40                  45

Pro Lys Thr Lys Arg Ser Pro Leu Thr Arg Ala His Leu Thr Glu Val
    50                  55                  60

Glu Ser Arg Leu Glu Arg Leu Glu Gln Leu Phe Leu Leu Ile Phe Pro
65                  70                  75                  80

Arg Asp Gln Lys Lys Phe Asn Lys Val Arg Val Val Arg Ala Leu Asp
                85                  90                  95

Ala Val Ala Leu Pro Gln Pro Val Gly Val Pro Asn Glu Ser Gln Ala
            100                 105                 110

Leu Ser Gln Arg Phe Thr Phe Ser Pro Gly Gln Asp Ile Gln Leu Ile
        115                 120                 125

Pro Pro Leu Ile Asn Leu Leu Met Ser Ile Glu Pro Asp Val Ile Tyr
    130                 135                 140

Ala Gly His Asp Asn Thr Lys Pro Asp Thr Ser Ser Ser Leu Leu Thr
145                 150                 155                 160

Ser Leu Asn Gln Leu Gly Glu Arg Gln Leu Leu Ser Val Val Lys Trp
                165                 170                 175

Ser Lys Ser Leu Pro Gly Phe Arg Asn Leu His Ile Asp Asp Gln Ile
            180                 185                 190

Thr Leu Ile Gln Tyr Ser Trp Met Ser Leu Met Val Phe Gly Leu Gly
        195                 200                 205

Trp Arg Ser Tyr Lys His Val Ser Gly Gln Met Leu Tyr Phe Ala Pro
    210                 215                 220

Asp Leu Ile Leu Asn Glu Gln Arg Met Lys Glu Ser Ser Phe Tyr Ser
225                 230                 235                 240

Leu Cys Leu Thr Met Trp Gln Ile Pro Gln Glu Phe Val Lys Leu Gln
                245                 250                 255

Val Ser Gln Glu Glu Phe Leu Cys Met Lys Val Leu Leu Leu Leu Asn
            260                 265                 270

Thr Ile Pro Leu Glu Gly Leu Arg Ser Gln Thr Gln Phe Glu Glu Met
        275                 280                 285

Arg Ser Ser Tyr Ile Arg Glu Leu Ile Lys Ala Ile Gly Leu Arg Gln
    290                 295                 300

Lys Gly Val Val Ser Ser Ser Gln Arg Phe Tyr Gln Leu Thr Lys Leu
305                 310                 315                 320
```

```
Leu Asp Asn Leu His Asp Leu Val Lys Gln Leu His Leu Tyr Cys Leu
                325                 330                 335

Asn Thr Phe Ile Gln Ser Arg Ala Leu Ser Val Glu Phe Pro Glu Met
            340                 345                 350

Met Ser Glu Val Ile Ala Gly Ser Thr Pro Met Glu Phe Gln Tyr Leu
        355                 360                 365

Pro Asp Thr Asp Asp Arg His Arg Ile Glu Glu Lys Arg Lys Arg Thr
    370                 375                 380

Tyr Glu Thr Phe Lys Ser Ile Met Lys Lys Ser Pro Phe Ser Gly Pro
385                 390                 395                 400

Thr Asp Pro Arg Pro Pro Pro Arg Arg Ile Ala Val Pro Ser Arg Ser
                405                 410                 415

Ser Ala Ser Val Pro Lys Pro Ala Pro Gln Pro Tyr Pro Phe Thr Ser
            420                 425                 430

Ser Leu Ser Thr Ile Asn Tyr Asp Glu Phe Pro Thr Met Val Phe Pro
        435                 440                 445

Ser Gly Gln Ile Ser Gln Ala Ser Ala Leu Ala Pro Ala Pro Pro Gln
    450                 455                 460

Val Leu Pro Gln Ala Pro Ala Pro Ala Pro Ala Pro Ala Met Val Ser
465                 470                 475                 480

Ala Leu Ala Gln Ala Pro Ala Pro Val Pro Val Leu Ala Pro Gly Pro
                485                 490                 495

Pro Gln Ala Val Ala Pro Pro Ala Pro Lys Pro Thr Gln Ala Gly Glu
            500                 505                 510

Gly Thr Leu Ser Glu Ala Leu Leu Gln Leu Gln Phe Asp Asp Glu Asp
        515                 520                 525

Leu Gly Ala Leu Leu Gly Asn Ser Thr Asp Pro Ala Val Phe Thr Asp
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Leu Ala Ser Val Asp Asn Ser Glu Phe Gln Gln Leu Leu Asn Gln Gly
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Ile Pro Val Ala Pro His Thr Thr Glu Pro Met Leu Met Glu Tyr Pro
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Glu Ala Ile Thr Arg Leu Val Thr Gly Ala Gln Arg Pro Pro Asp Pro
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Ala Pro Ala Pro Leu Gly Ala Pro Gly Leu Pro Asn Gly Leu Leu Ser
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<223> Six repeating GAL-4 DNA binding site and a TATA box.

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tcgagggtcg aagcggagta ctgtcctccg agtggagtac tgtcctccga gcggagtact 120

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tgaaccgtc 189

<210> 19
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<223> Core promoter region comprising TATA box, (16) ... (23), a putative initiation region (inr), (35) ... (64), from the CMV promoter, and 5'UTR, (59) ... (175) from CMV.

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ccagcctccg cggccgggaa cggtgcattg gaacgcggat tccccgtgtt aattaa 176

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caccgcttcg tccttaacgg gcccggcggt gccgggggat tatttcggcc ccggccccgg 240

gggggcccgg cagacgctcc ttatacggcc cggcctcgct cacctgggcc gcggccagga 300

gcgccttctt tgggcagcgc cgggccgggg ccgcgccggg cccgacaccc aaatatggcg 360

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ccggggccgg cgggcgact 439

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gcgctctaaa aataactccc gggagttatt tttagagcgg tgaggaatgg tggacaccca 180

aatatggcga cggaaatatg gcgacggcac cattcctcac gacacccaaa tatggcgacg 240

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<211> 207
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<223> Muscle specific transcriptional regulating region MEF-1.

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<223> Muscle specific transcriptional regulating region MEF-2.

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<223> Muscle specific transcriptional regulating region TEF-1.

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<223> Muscle specific transcriptional regulating region SP1.

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<223> Complete nucleotide sequence of GeneSwitch plasmid pGS1633.

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tctgacagaa gtggaatcaa ggctagaaag actggaacag ctatttctac tgatttttcc 300

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cccacagcca gtgggcgttc caaatgaaag ccaagcccta agccagagat tcacttttc 420

accaggtcaa gacatacagt tgattccacc actgatcaac ctgttaatga gcattgaacc 480

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gagcttaatg gtgtttggtc taggatggag atcctacaaa cacgtcagtg ggcagatgct 720

gtattttgca cctgatctaa tactaaatga acagcggatg aaagaatcat cattctattc 780

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aagtcaaacc cagtttgagg agatgaggtc aagctacatt agagagctca tcaaggcaat 960

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tcttgataac ttgcatgatc ttgtcaaaca acttcatctg tactgcttga atacatttat 1080

ccagtcccgg gcactgagtg ttgaatttcc agaaatgatg tctgaagtta ttgctgggtc 1140

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Glu Arg Asn Gln Glu Gln Gly Ala
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/17573

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12N 15/63
US CL : 435/320.1, 536/23.1, 24.1
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/320.1, 536/23.1, 24.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, EMBASE, CAPLUS, SCISEARCH, BIOSIS, REG, GENBANK

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95/18380 A1 (The Salk Institute for Biological Studies) 06 July 1995 (06.07.95), see entire reference.	1-10, 18, 23-27, 34
X --- A	WO 93/09236 A1 (Baylor College of Medicine) 13 May 1993 (13.05.93), see entire reference.	1 --- 34-44
A	WO 88/10307 A1 (Gentech, Inc.) 29 December 1988 (29.12.88), see entire reference.	37-44

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 September 2001 (10.09.2001)	08 FEB 2002
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Lauren Nguyen, Ph.D. Telephone No. 703-308-0196

Form PCT/ISA/210 (second sheet) (July 1998)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/17573

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-33 and 44, drawn to an inducible GHRH expression system, wherein the special technical feature is an inducible GHRH expression system comprising a first expression cassette encoding a ligand specific molecular switch protein and a second expression cassete encoding a promoter and a protein having a GHRH activity to induce production of IGF-1. Group II, claims 34 and 35, wherein the special technical feature is an inducible expression system comprising a first expression cassete having a tissue-specific promoter and a second expression cassette encoding a protein having GHRH activity and a ligand capable of binding to a mutated ligand-binding domain. Group III, claim 36, wherein the special technical feature is a GHRH inducible expression system comprising two plasmids having SEQ ID Nos. 28 and 29. Group IV, claim 37, wherein the special technical feature is a composition comprising a pharmacological dose of two plasmids each of which has different expression cassettes. Group V, claims 38 and 39, wherein the special technical feature is a method of inducing the expression of a GHRH transgene. Group VI, claims 40 and 41 wherein the special technical feature is a method for regulated GHRH expression in vivo for use in indications selected from the group consisting of: increasing weight, increasing lean body mass, decreasing fat mass, conversion to anabolsim for a catabolic state associated with wasting and increasing bone area, content and density. Group VII, claims 42 and 43, wherein the special technical feature is a method of regulated exposure to GHRH in vivo comprising adminstering a regulated GHRH gene expression system and adminstering a pharmacologic dose of mifepristone on a pulsatile schedule.

These inventions listed as Groups do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical feature as to form a single general inventive concept.